A Study of the Biogenesis of Secreted Proteins in *Saccharomyces cerevisiae.*

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I dedicate this thesis to Mr and Mrs T. Whitley, "Mum and Dad"
for their support and encouragement over the last 24 years
which has made this thesis possible.

Declaration.

This study was carried out under the supervision of Dr. Alan Boyd in the Department of Biochemistry, University of Edinburgh between September 1987 and December 1990.

The experimental work presented in this thesis, unless stated otherwise, is my own; and this manuscript presented here has been composed by myself.

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Abstract.

The maturation of many secreted proteins in *Saccharomyces cerevisiae* involves the cleavage of a precursor polypeptide after pairs of basic residues by a specific protease (Kex2p) at a late stage in the secretory pathway. The aim of this project was to determine the order of processing events and the importance of ordered processing in the maturation of a multi-subunit protein toxin (the *Kluyveromyces lactis* killer toxin) in the *S. cerevisiae* secretory pathway. The involvement of Kex2p in the maturation of the *K. lactis* killer toxin was confirmed and the cleavage event was shown to most likely occur at a late stage in the secretory pathway. In order to determine whether the timing of Kex2 processing was critical for efficient maturation and secretion of secretory proteins an attempt was made to localise the Kex2 activity to the endoplasmic reticulum (ER) and thus cause premature Kex2 processing of secretory proteins. In order to localise Kex2 activity to the ER, various constructs were made based on an active portion of the Kex2p that can be secreted. Various sequences were fused to the C-terminus of this truncated Kex2p in an attempt to localise Kex2 activity to the ER. A truncated Kex2 polypeptide with the 60 C-terminal amino acids of yeast protein disulphide isomerase (PDI) added to create a Kex2-PDI hybrid protein was more efficiently retained within the cell than any of the other constructs, including a truncated Kex2p with the yeast HDEL retention signal added to the C-terminus. The implications of these results are discussed in the thesis.

Analysis of the effect of the different Kex2 constructs on the processing and assembly of the *K. lactis* killer toxin was hampered by the complexities of the toxin system and technical difficulties. Therefore in order to determine the activities of the various Kex2 constructs *in vivo* the production of the peptide mating pheromone α factor was measured. Suprisingly the efficiently retained Kex2-PDI hybrid protein exhibited the lowest *in vivo* activity in this experiment. Assuming that the Kex2-PDI hybrid protein is localised to the ER as predicted, the results suggest that Kex2p localised in the ER cannot facilitate the efficient maturation of secretory proteins into an active secreted form. Possible reasons for this are discussed in the thesis.

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Abbreviations

AMC	7-amino-4-methylcoumarin
ATP	adenosine 5'-triphosphate
bp	base pair
bQRR-MCA	t-butoxycarbonyl-Gln-Arg-Arg 4-methylcoumarin-7-amide
CHO	Chinese hamster ovary
Ci	Curie
CPY	carboxypeptidase Y
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	diaminoethanetetra-acetic acid
ER	endoplasmic reticulum
hr	hour
IPTG	isopropylthiogalactoside
K	kilodalton
kb	kilobase
min	minute
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second
SRP	signal recognition particle
SV	secretory vesicle
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol(tris)
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactoside

CONTENTS

List of figures.

List of tables

Chapter one.

Introduction.

1. INTRODUCTION

In eukaryotic cells, secretory proteins must be accurately transported between a series of membrane bounded organelles, and eventually to the cell surface where they are released. Secretory proteins released at the cell surface differ from the cognate primary translation products due to modifications made to the proteins in the secretory pathway during transport. The following sections attempt to give a broad view of the eukaryotic secretory pathway and processes that take place in this pathway based on current knowledge. Aspects of the secretory pathway particularly relevant to this project will be discussed in greater detail. Secretion of proteins from the yeast *Saccharomyces cerevisiae* will be discussed separately as it is the experimental organism used in this project.

1.1 The secretory pathway of mammalian cells: a brief overview.

Proteins that enter the secretory pathway are synthesised on polysomes attached to the rough endoplasmic reticulum (RER) (Siekevitz and Palade 1960) and are translocated across the endoplasmic reticulum (ER) membrane. Translocated secretory proteins move from the ER to the Golgi complex where they are packaged into secretory vesicles and transported to the plasma membrane. As well as proteins destined for the cell surface and secretion, other classes of protein enter the secretory pathway, both soluble and membrane bound. Lysosomal proteins and proteins resident in the different organelles of the secretory pathway all enter the secretory pathway in the same way as secreted proteins, but do not usually reach the cell surface. The correct localisation of proteins that enter into the secretory pathway is necessary to maintain the integrity and function of the cell.

1.1.1 Translocation of proteins across the ER membrane.

Proteins to be translocated across the ER membrane possess a specific signal sequence, usually located at the extreme amino terminus of the protein (Blobel and Dobberstein 1975). There is no consensus signal sequence but a number of features are commonly found. They are:- the presence of 1-3 positively charged amino acids in the amino terminal region, followed by a long hydrophobic sequence consisting of 14-20 amino acids and ending in a glycine or an alanine at the carboxy terminus of the signal peptide (von Heijne 1985). The signal sequence of a nascent polypeptide, upon emergence from the ribosome, is recognised by the signal recognition particle (SRP) (Walter and Blobel 1981) through its 54K component (Krieg *et al* 1986). In the absence of membranes SRP can induce an arrest of elongation of the polypeptide *in vitro* (Walter and Blobel 1981). Interaction of SRP with its receptor in the ER membrane (docking protein) (Meyer et al 1982, Gilmore *et al* 1982) causes SRP to be released from the ribosome (Gilmore and Blobel 1983) and from the signal sequence (Wiedmann *et al* 1987a). The signal sequence is then found in the close proximity of a 35K integral glycosylated membrane protein of the ER (Wiedmann *et al* 1987b). The actual process of passage of the polypeptide across the phospholipid bilayer is still poorly understood. A ribosome receptor and a proteinaceous tunnel to provide a hydrophilic environment for the passage of the polypeptide chain have been postulated (Blobel and Dobberstein 1975) but not yet identified. Proteins will usually pass through the ER membrane into the lumen of the ER unless a second hydrophobic region which can anchor the protein in the membrane is present (for review see Rapoport and Weidmann 1985).

1.1.2 Modifications in the ER.

Polypeptides translocated across the ER membrane are acted upon by a number of enzymes on the luminal side of the membrane. As the protein enters the lumen of the ER the signal sequence is usually removed by signal peptidase (Baker *et al* 1986, Evans *et al* 1986). A high mannose oligosaccharide core unit is transferred to selected asparagine residues located in the sequence Asn-X-Ser/Thr where X can be any amino acid except proline (see fig. 1.1). The efficiency of this N-linked glycosylation is dependent on the conformation of the peptide chain as it enters the lumen of the ER, as potential glycosylation sites may no longer be accessible once the protein has folded (for review see Kornfeld and Kornfeld 1985). Some modifications

to the N-linked oligosaccharide occur in the ER (see fig. 1.1 and section 1.1.5). Another type of modification that takes place in the ER is the formation of disulphide bonds by thiol oxidation and disulphide exchange which is catalysed by the enzyme protein disulphide isomerase (PDI) (Freedman 1984). PDI has also been implicated in a number of other processes that take place in the ER. As well as existing as a free monomer, PDI is an essential subunit of prolyl-4-hydroxylase, an enzyme that catalyses the modification of prolyl residues in newly synthesised collagen (Pihlajaniemi *et al* 1987). PDI also binds to the Asn-X-Ser/Thr acceptor sequence for N-linked glycosylation and is an important component of oligosaccharide transferase (Geetha Habib *et al* 1988). As well as making these covalent modifications to polypeptides, proteins present in the ER may be involved in helping proteins to fold and assemble correctly (see later sections).

1.1.3 Transport between the ER and the Golgi

Electron microscopic studies have suggested that small vesicles transport proteins from the RER to the Golgi apparatus. Regions of the RER free of ribosomes are thought to be nascent transport vesicles but may also represent vesicles that are fusing with the ER (Farquarh and Palade 1981, Saraste and Kuismanen 1984). Vesicles responsible for transport of proteins between the ER and Golgi have been described biochemically (Lodish *et al* 1987). More recently, cell-free systems have been developed that reconstitute ER to Golgi transport of newly synthesised glycoproteins (Haselback and Schekman 1986, Balch *et al* 1987). In these cell-free systems, movement to the Golgi apparatus is monitored by the action of a Golgi oligosaccharide processing enzyme on the glycoprotein from the donor ER vesicles. It has been discovered that for the donor glycoprotein to be modified by the Golgi enzymes, energy in the form of ATP, cytosolic proteins and membrane proteins are required. However, in these cell free systems vesicular intermediates of transport between the ER and Golgi have not been detected and promiscuous fusion of ER and Golgi vesicles may be occurring that does not occur *in vivo*.

1.1.4 The Golgi complex.

The Golgi complex consists of at least three functionally and compositionally distinct compartments (or cisternae) that operate in succession to construct the oligosaccharide chains on transported glycoproteins (see fig. 1.1)(Dunphy and

Rothman1985) and sort out proteins destined for lysosomes, the cell surface or secretory granules. The Golgi complex is also the site of processes such as O-linked glycosylation (for review see Kornfeld and Kornfeld 1985), sulphation (Huttner 1988), phosphorylation of lysosomal enzymes (von Figura and Hasilik 1986) and proteolytic processing of prohormones (for review see Thomas *et al* 1988).

Transport between adjacent cisternae of the Golgi complex is thought to be mediated by the budding and fusion of vesicles (for review see Pfeffer and Rothman 1987) that requires energy and protein components in a cell-free system (Malhotra *et al* 1988) in a similar fashion to transport between the ER and Golgi apparatus. Vesicular transport between membrane bounded compartments in a cell allows the compartments to be separate from one another and able to contain different intra-compartmental environments. Conditions such as pH, ionic strength and protein content can be varied from compartment to compartment. This compartmentalisation of organelles allows the wide variety of modifications that are needed for the maturation of proteins in the secretory pathway to occur in an ordered fashion.

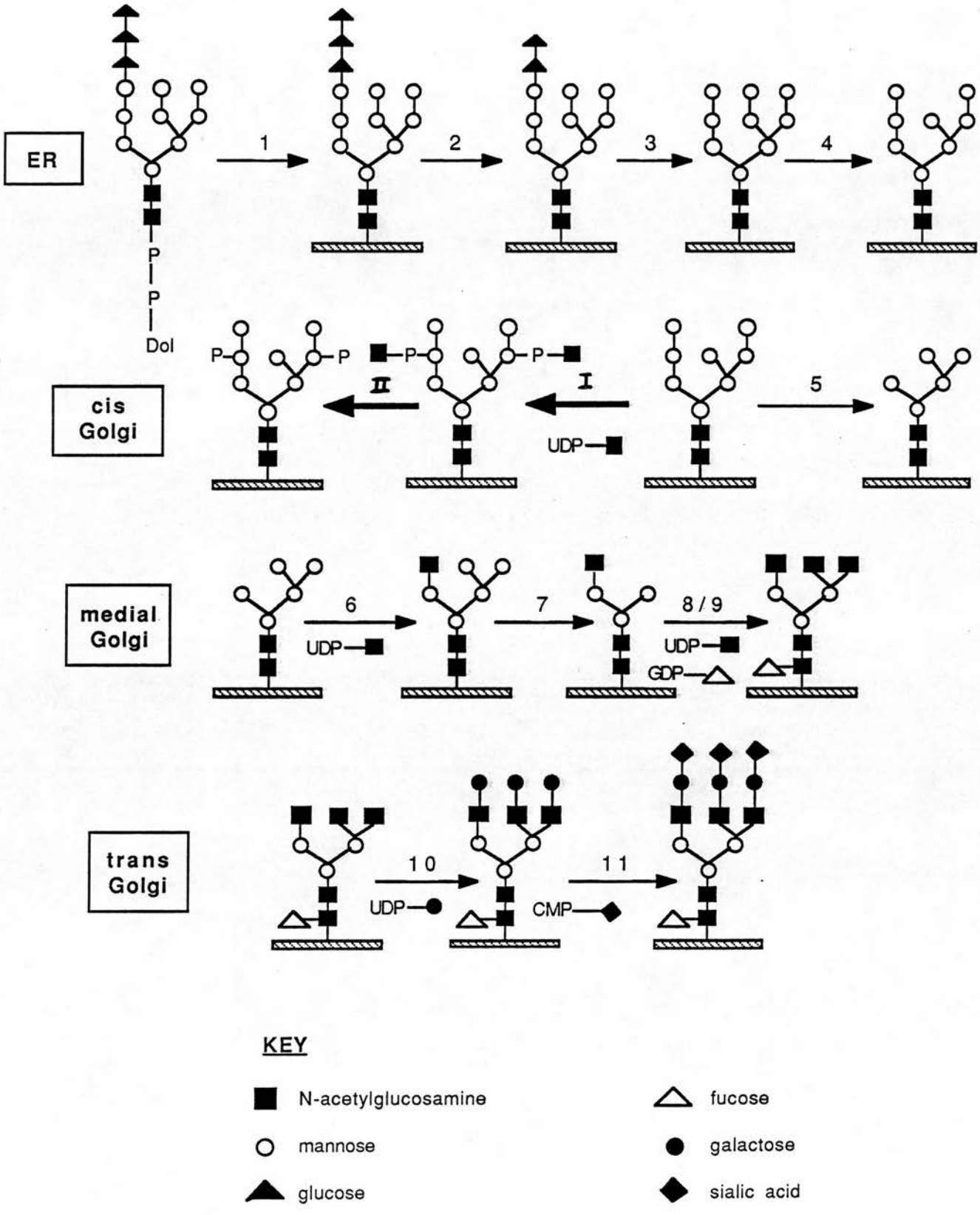
1.1.5 N-linked glycosylation.

Most proteins that are synthesised in the ER become glycosylated. The elaborate series of covalent modifications that take place in the ER and Golgi apparatus are well established and the structure of the oligosaccharide moiety indicates to which enzymes in the processing pathway the glycoprotein has been exposed. In many cases this information can be used to infer which compartment the glycoprotein has reached during transport through the secretory pathway (for review see Kornfeld and Kornfeld 1985)

The first step in the synthesis of N-linked oligosaccharides is the transfer of the precursor $Glc_3Man_9GlcNAc_2$ (Glc = glucose, Man = mannose, and GlcNAc = N-acetylglucosamine) from its lipid donor to an asparagine residue on a nascent polypeptide in the RER, (fig. 1.1: reaction 1). The terminal Glc is removed (reaction 2) and then the two inner Glc residues are removed (reaction 3). The specific glucosidases that catalyse these reactions are located in the membranes of the RER, as is a specific α–mannosidase which catalyses the removal of at least one α1,2-linked

Fig. 1.1 N-linked glycosylation.

This figure is a diagrammatic representation of N-linked glycosylation through the secretory pathway. The enzymes that catalyse the reactions are:- oligosaccharyltransferase (1), α-glucosidase I (2), α-glucosidase II (3), ER 1,2 mannosidase (4), N-acetylglucosaminylphosphotransferase (I), N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (II), Golgi α-mannosidase I (5), N-acetylglucosamiyltransferase I (6), Golgi α-mannosidase II (7), N-acetylglucos--aminyltransferase II (8), fucosyltransferase (9), galactosyltransferase (10) and sialyltransferase (11). See text and review by Kornfeld and Kornfeld (1985) for further details.


ER
cis Golgi
medial Golgi
trans Golgi
Dol
P
1
2
3
4
5
6
7
8 / 9
10
11
I
II
UDP
GDP
CMP
KEY
N-acetylglucosamine
mannose
glucose
fucose
galactose
sialic acid

Man residue (reaction 4). Once transported to the Golgi apparatus, further modifications take place. In the *cis*-Golgi, lysosomal enzymes undergo a specific Man phosphorylation (reactions I and II). The high Man oligosaccharides on non-lysosomal glycoproteins can be further trimmed by a Golgi mannosidase (reaction 5) to yield a $Man_5GlcNAc_2$ structure in the *cis* or medial Golgi cisternae. In the medial cisternae oligosaccharides destined to become complex type structures are processed by the addition of GlcNAc (reaction 6) followed by the removal of two Man residues (reaction 7) and subsequent addition of another GlcNAc residue (reaction 8). At this stage fucose may be added to the innermost GlcNAc residue (reaction 9). In the trans-Golgi outer chain galactose and sialic acid residues are added (reactions 10 and 11). Other terminal sugar additions are also thought to occur at this late stage.

1.1.6 Proteolytic processing

Small peptide hormones and neurotransmitters are usually synthesised as larger precursor proteins that undergo proteolytic cleavage to produce mature bioactive peptides (Thomas *et al* 1988). The peptides in precursor proteins are usually flanked by pairs of basic residues ie Lys-Lys, Arg-Arg or Lys-Arg and diverse enzymes have been implicated in the processing at such sites (Loh *et al* 1987, Clamagrand *et al* 1987, Gluschankof 1987 , Davidson 1987, Marx 1987). Although proteases with the required specificities for cleavage have been isolated from mammalian cells their exact physiological roles have not been identified. The Kex2 protease of the yeast *Saccharomyces cerevisiae* has been much studied and has been shown to have a physiological role in prohormone processing which will be discussed in later sections. After cleavage at pairs of basic amino acid residues, flanking amino acids are usually removed by a carboxypeptidase B-like enzyme or a similar aminopeptidase (Thomas *et al* 1988).

1.1.7 The problem of protein sorting

The problem of how proteins in eukaryotic cells reach their final destinations so that the subcellular organelles of a cell can be formed and maintained has been the subject of a lot of recent attention. A pattern is emerging to suggest that the proteins themselves contain the signals that direct them to the appropriate locations in the

cell. As has already been mentioned, proteins that are to be translocated across the ER membrane contain structural motifs that facilitate this transfer (von Heijne 1985). Similarly mitochondrial proteins contain sequences that allow them to be incorporated into this organelle (Horwich *et al* 1985, Schatz 1987). The targetting sequences mentioned above are primarily located in the amino-terminal (N-terminal) region, but other targetting sequences may occur elsewhere in the protein. The structural motifs that allow a protein to enter into the nucleus are short linear sequences located at internal positions within the protein (Dingwall *et al* 1982, Kalderon *et al* 1984). Also the attachment of mannose-6-phosphate, a signal required for transport of proteins to the lysosome in mammalian cells is not necessarily confined to the N-terminal end (Kornfeld 1986, von Figura and Hasilik 1986) and peroxisomal targetting sequences have been identified in the carboxy terminal (C-terminal) region of a protein (Miyazawa *et al* 1989, Gould *et al* 1989).

Proteins that are destined for secretion, and proteins that constitute structural and functional components of the secretory organelles have somehow to be distinguished from one another as they all enter the secretory machinery by being translocated across the ER membrane. It was originally suggested that proteins destined for secretion contained specific sequences that targetted them to be secreted (Lodish *et al* 1983). However, the view now more widely held is that secretion occurs by a bulk flow mechanism and proteins that are to be prevented from being secreted are somehow selectively retained.

One striking piece of evidence to suggest that secretion occurs by default comes from experiments performed by Wieland *et al* (1987). Their experiments showed that a synthetic tripeptide consisting of the consensus glycosylation sequence Asn-Tyr-Thr, esterified to make it membrane permeable can enter cells, be glycosylated in the ER (thus making it membrane impermeant) and can then be rapidly secreted. Furthermore the data from these experiments suggests that at least some of the secreted tripeptide passes through the normal secretory and the Golgi apparatus, as the appropriate modifications to the attached oligosacccharide take place.

1.1.8 Retention of proteins

Resident proteins of the various secretory organelles must somehow avoid export by the bulk flow pathway of secretion. It is easy to imagine how membrane proteins that are residents of secretory organelles may escape export by bulk flow by being held in position by interactions between their cytoplasmic domains and other cellular structures. In fact it has been shown that short cytoplasmic sequences serve as retention signals for transmembrane proteins in the ER , (Nillson *et al* 1989, Poruchynsky and Atkinson 1988) although the proteins with which they interact have not yet been identified. For truly soluble proteins in the lumen of secretory organelles, retention represents a greater conceptual problem. If these proteins are soluble and easily diffusible it would seem that they will tend to be secreted by the bulk flow as are secretory proteins. There must therefore be some way of preventing this class of proteins from being secreted if the integrity and function of the cell is to be maintained.

The following sections attempt to describe mechanisms by which proteins may be localised to organelles by specific and non-specific means. The organelle that will be concentrated upon is the ER, as the majority of work has been carried out on ER retention.

1.1.9 Different proteins are secreted at different rates.

Although secretory proteins are thought to be secreted by a bulk flow default pathway, different proteins are secreted at different rates from the cell. The halftime of exit of proteins from the ER varies from about 15 minutes for some viral glycoproteins (Quinn *et al* 1984 , Copeland *et al* 1988) and some serum proteins to 2 hours or more for some other serum proteins (Fries *et al* 1984 , Lodish *et al* 1983, Yeo *et al* 1985). Wieland et al estimated the halftime for the secretion of the synthetic tripeptide Asn-Tyr-Thr from tissue culture cells to be about 10 minutes, although whether this is a true reflection of the rate of bulk flow has been questioned (Rose and Doms 1988). However it seems reasonable to assume that the bulk flow rate is close to the rate of transport of the most rapidly secreted proteins as no positive secretion signals have been found for any protein. The most likely reason why specific proteins are secreted more slowly than expected from the rate of bulk flow is that these proteins are retarded in their progress through the

secretory pathway by interactions with other elements of the pathway, with the ER appearing to be the organelle in which the majority of such interactions take place. Some of these interactions will be discussed in the following sections.

1.1.10 Protein folding and assembly in the ER.

The correct folding of monomeric proteins and the correct folding and assembly of multimeric proteins is necessary for their efficient transport to the cell surface (for review see Hurtley and Helenius 1989). This is probably because unfolded proteins tend to bind other proteins in the ER or form insoluble aggregates that are unable to enter transport vesicles. The folding of proteins *in vitro* is often slow and inefficient but proteins fold *in vivo* into the correct conformation rapidly and efficiently due, at least in part to several resident ER proteins. In addition these resident ER proteins may bind to aberrantly assembled proteins to prevent their export. For example protein disulphide isomerase (PDI) interacts with unfolded proteins and catalyses the formation of disulphide bonds, which are often required for the correct folding of a protein. Other proteins such as CD-ω or TRAP (Alarcon et al 1988, Bonifacino et al 1988), the collagen binding protein colligin (Hughes et al 1987) and the abundant ER protein GRP94 (Sorger and Pelham 1987, Lee 1987) may also have roles to play in protein assembly and the prevention of premature export of proteins. However, perhaps the protein most widely implicated in protein folding and assembly *in vivo* is BiP (also known as GRP78). The following sections discuss the possible functions of BiP.

1.1.11 The role of BiP

A major resident ER protein is the soluble protein BiP which is also known as glucose-regulated protein (GRP78). BiP has been found tightly associated in the ER with newly synthesised proteins that are incompletely assembled, have mutant structures or are incorrectly glycosylated . BiP was originally found associated with unassembled immunoglobulin heavy chains before their assembly with light chains, thus preventing transport to the Golgi apparatus (Bole *et al* 1986). BiP can be released from its association with heavy chains *in vitro* by incubation in the presence of ATP (Munro and Pelham 1986). BiP is found bound to mutant or malfolded forms of influenza haemagglutinin (HA) (Gething *et al* 1986), to a

derivative of SV40 T antigen that is fused to a signal sequence to allow it to enter the ER (Sharma *et al* 1985), in an *in vitro* translation translocation system to unoxidised (but not to disulphide bonded) prolactin and to unglycosylated but not glycosylated invertase (Kassenbrock *et al* 1988). Proteins that are inhibited in glycosylation tend to fold poorly (Leavitt *et al* 1977) and BiP is found associated with many proteins that have had their glycosylation inhibited (Bole *et al* 1986, Gething *et al* 1986, Dorner *et al* 1987 , Hendershot *et al* 1988).

It has been suggested that the associations with BiP are all hydrophobic in nature (Pelham 1986 and 1989) which could mean that BiP recognises hydrophobic regions of misfolded proteins that are normally buried or masked by glycosylation in the correctly folded protein. However, in contrast with this view, it has been reported that both hydrophilic and hydrophobic peptides are bound by BiP (Flynn et al 1989). Many of the known substrates for BiP are aberrant proteins but BiP may also have a role to play in the correct maturation and folding of normal proteins in the secretory pathway. One example that has already been mentioned is the binding of BiP to immunoglobulin heavy chains that are not aberrant, before their assembly with light chains (Bole *et al* 1986). Also BiP has been observed bound transiently to several human serum glycoproteins expressed in CHO cells. The transient binding of BiP to secretory proteins is probably one of the reasons for the different half times of secretion for different proteins. Some proteins may stay in the ER longer than others because of difficulty in folding into a structure able to avoid the binding of BiP by hydrophobic interactions and therefore have a longer half time for secretion.

1.1.12 The action of BiP

The association of BiP with aberrant proteins and transiently with normal proteins has been described in the previous section but the mode of action has not been discussed . The immunoglobulin heavy chain binding protein BiP is a member of the evolutionary conserved HSP-70 family of heat shock proteins (Hutton *et al* 1987). Like hsp70, BiP binds ATP tightly and the hydrolysis of ATP allows it to be released from at least some of the substrates which it binds (Munro and Pelham 1986, Dorner *et al* 1987, Kassenbrock *et al* 1988). One model for the action of BiP and other members of the HSP-70 family is that they act as reversible detergents, binding to hydrophobic surfaces and using the energy from ATP hydrolysis to change

the conformation of the protein to a non-binding state (Pelham 1986). This would have the effect of keeping unfolded or unassembled proteins in solution, avoiding aggregation or precipitation into permanently non-functional complexes because of hydrophobic interactions, and thus allow them to achieve their final tertiary or quaternary structures by using the energy of ATP hydrolysis.

Although the main function of BiP is probably to promote the normal folding and assembly of proteins it may also rescue misfolded proteins from their misfolded fate by using the energy from ATP hydrolysis to change the proteins conformation, and thus allow them another chance to fold correctly. BiP may also function to stop the exit of misfolded proteins from the ER until they either fold correctly or are degraded. Association with BiP may be one of the reasons for the inefficient secretion of many proteins expressed in heterologous eukaryotic cells. In summary it appears that BiP has an important role to play in enabling proteins to reach a transport competent conformation and in preventing the exit of aberrant proteins.

1.1.13 Degradation of proteins in the ER.

Large numbers of proteins in the ER are transport-incompetent for some of the reasons mentioned previously, so the question arises of how the cell disposes of them as it would be reasonable to assume that the accumulation of aberrant proteins in the ER would be deleterious to the cell. Although misfolded proteins are inherently more sensitive to proteases than correctly folded proteins, they often have a relatively long half-life in the ER (Corless et al 1987, Hurtley et al 1989). Often a lag period followed by a rapid degradation phase is seen which possibly suggests that to be degraded, proteins need to be moved to a specific compartment away from the ER. It has been suggested that degradation of some unassembled and misfolded proteins probably occurs in the ER itself (Lippincott-Schwartz et al 1988), although the identity of the degradative enzymes in the ER is not known. Degradation of some other aberrant proteins may take place in lysosomes as their degradation is blocked by inhibitors of lysosomal degradation (Davis and Hunter 1987). Further studies are needed to determine where and how the degradation of the wide variety of retained proteins occurs and how these processes are controlled.

1.1.14 Retention of luminal ER proteins

The previous sections have described how aberrant proteins and proteins en route through the secretory pathway may be held up in the ER, but there are proteins in the lumen of the ER that are resident there and necessary for its function. A mechanism must therefore exist to prevent these luminal ER proteins from leaving the cell with other secretory proteins.

The first indication that a specific mechanism for the retention of luminal ER proteins came from the observation that that PDI and BiP have a common C-terminal tetrapeptide, KDEL, (Lys-Asp-Glu-Leu) (Munro and Pelham 1986). It has now been determined that luminal ER proteins from a variety of species have this C-terminal sequence or a related sequence such as HDEL (*Saccharomyces cerevisiae*) (Rose *et al* 1989) RDEL (rat colligin), KEEL (mouse ERp72) (Pelham 1989) or DDEL (*Kluyveromyces lactis*) (Lewis *et al* 1990) . Although there is some divergence in the C-terminal sequence in different organisms, the similarity suggests that these C-terminal tetrapeptides may act as retention signals for the ER.

The importance of the KDEL sequence for retention of proteins in the ER has been shown in experiments in which altered proteins were expressed in COS cells. The removal of KDEL from BiP caused it to be slowly secreted from COS cells and the addition of the KDEL tetrapeptide to the C-terminus of lysozyme (a protein that is normally secreted) caused it to accumulate in the ER of COS cells (Munro and Pelham 1987). Furthermore experiments in which the C-teminus of lysozyme was changed to KDAS or KDELGL resulted in the secretion of lysozyme suggesting that KDEL is required at the extreme C-terminus of a protein for the protein to be efficiently retained.

The addition of the SEKDEL sequence to the C-termini of two secretory proteins, rat growth hormone (rGH) and human chorionic gonadotrophin (hCGa), does not prevent them from being secreted but only retards the proteins in their progress through the secretory pathway. Thus it seems as if the addition of the C-terminal KDEL sequence is sufficient to prevent the secretion of certain proteins but for others it merely increases the half time for secretion (Zagouras and Rose 1989). In either case it appears that KDEL does have a role to play in at least slowing the exit from the cell of proteins containing this sequence.

1.1.15 How does the KDEL sequence function?

Experimental data suggests that the KDEL sequence is involved in the specific retention of resident ER proteins, so the question is "how is this sequence promoting the retention of these proteins?" One simple explanation of the function of the C-terminal KDEL sequence could be that the sequence is recognised by an integral membrane protein (a KDEL receptor) present in the ER, thus preventing exit of proteins containing this sequence. However, KDEL-containing proteins are very abundant in the ER and no membrane protein has been described that is present in sufficient quantity to bind all of the KDEL-containing proteins and immobilise them in the ER. To try to explain the apparent excess of KDEL-containing proteins compared to putative receptor, a model was proposed in which KDEL receptors captured proteins with the C-terminal KDEL sequence from a post-ER salvage compartment and then returned them to the ER (Warren 1987, Munro and Pelham 1987). The receptor would not need to be present in stoichiometric amounts as it would not be functioning to bind the KDEL containing proteins permanently but would only associate with them transiently until the KDEL-containing protein was returned to the ER.

One piece of evidence that suggests that KDEL-containing proteins are not continuously and tightly bound to a component of the ER comes from micro-injection experiments (Ceriotti and Coleman 1988). These experiments show that the presence of a KDEL tail on BiP does not significantly alter its rate of diffusion in the ER compared with that of BiP bearing the unrecognised KDELGL C-terminus. Both of the BiP constructs diffuse more rapidly than an integral membrane protein haemaglutinin but more slowly than albumin, a protein that is secreted. It has been shown that proteins with the KDEL signal can leave the ER transiently since the modification of the oligosaccharide side chains by the addition of UDP-GlcNAc to mannose residues, occurs in a cathepsin D (normally a lysosomal protein) hybrid protein bearing the C-terminal KDEL sequence (Pelham 1988). The phosphotransferase that catalyses the addition of UDP-GlcNAc is thought to reside not in the ER but in a smooth-membraned compartment close to, but possibly distinct from the *cis* Golgi. The KDEL-containing cathepsin D was not transported to lysosomes as normal cathepsin D would be, but was found to have accumulated in the ER. The removal of the GlcNAc from the oligosaccharide (see fig. 1.1) of the

cathepsin D hybrid was not observed, suggesting that the hybrid protein does not penetrate far into the Golgi stack before being returned to the ER. The oligosaccharides on GRP94, glucosidase II and of colligin which are all thought to be luminal ER proteins are of high mannose type and do not appear to have been exposed to the Golgi mannosidase I which is thought of as a cis Golgi marker. Thus it appears that separation of resident ER proteins may occur in a salvage compartment that contains phosphotransferase but lacks N-acetylglucosaminidase and Golgi mannosidase I.

For the retrieval model to be correct the putative salvage compartment should have a luminal environment distinct from that of the ER, one that promotes the binding of KDEL to its receptor. Conversely the environment of the ER should allow the release of the KDEL-containing protein by its receptor, thus allowing the receptor to return to the salvage compartment to recover more KDEL-containing protein. Many of the organelles in a cell contain distinct proteins that are considered to be markers of that particular organelle. There are now possible marker proteins described for the salvage compartment. A 58K glycoprotein has been localised by immunoelectron microscopy to the *cis* face of the Golgi, to the vesicles and tubules associated with it and occasionally to the adjacent transitional regions of the ER (Saraste *et al* 1987). A 53K non-glycosylated protein that exists as a hexamer has also been found localised to smooth vesicles and tubules between the ER and the Golgi (Schwiezer *et al* 1988). These two proteins are both membrane proteins and are possible candidates for markers of the putative salvage compartment.

1.1.16 The recycling mechanism.

It has recently been shown that the movement of proteins between the salvage compartment and the ER is probably mediated by vesicular movement along microtubules. This return to the ER from post ER compartments has been termed retrograde transport as movement is in the opposite direction from that associated with secretion (Lippincott-Schwartz *et al* 1990). The involvement of microtubules in retrograde transport has been shown by experiments performed by Lippincott-Schwartz *et al* (1990). A temperature block results in the accumulation of a unique 53K antigen in the salvage compartment. At 16^{o}C retrograde transport back to the ER is inhibited while flow from the ER to the salvage compartment continues. At 16^{o}C the antigen can be seen to be leaving the salvage structures

identical to those seen in experiments using Brefeldin A to fuse the salvage compartment to the Golgi cisternae (Lippincott-Schwartz *et al* 1990). Electron microscopy shows that the necklaces consist of vesicles and tubules that are being transported along microtubules. If the temperature block is released the 53K protein leaves the salvage compartment and can be transiently detected in the ER . Treatment of cells with microtubule depolymerising drugs inhibits the flow of the 53K antigen from the salvage compartment to the ER but not from the ER to the Golgi.

1.1.17 The KDEL receptor.

Conventional approaches to identifying the KDEL receptor using affinity columns did not prove to be encouraging. An alternate approach of generating anti idiotypic antibodies has proved to be more promising (Vaux *et al* 1990). The strategy in raising anti idiotypic antibodies to the KDEL receptor is as follows;- antibodies are raised against peptides containing the KDEL signal. Antibodies against the KDEL binding site of these anti KDEL antibodies can then be raised. If the KDEL binding site of the anti KDEL antibodies mimics the binding site of the native KDEL receptor, the antibodies raised against the KDEL binding sites of these anti KDEL antibodies should also recognise the native KDEL receptor. Anti idiotypic antibodies raised using three slightly different variations of the above strategy (Vaux *et al* 1990) have been found to recognise a 72 K integral membrane protein in all vertebrate species tested. To show that the 72 K protein is likely to be the KDEL receptor, it has been shown that antibodies raised against the anti 72K protein antibodies can recognise peptides ending in KDEL. It therfore appears as if the 72K protein is a good candidate for the KDEL receptor.

When used to examine the subcellular location of the KDEL receptor the anti idiotypic antibodies showed the same distribution as the salvage compartment markers (described in section 1.1.15) and the redistribution of the KDEL receptor when a 16^{o}C temperature block is introduced is the same. These observations suggest further that the 72K protein is the KDEL receptor and is recycled between the ER and the salvage compartment.

1.1.18 Is the C-terminal KDEL sequence sufficient to localise proteins to the ER :-The possible role of calcium.

Although the KDEL signal has been shown to be involved in the recycling of proteins to the ER, it cannot be ruled out that other mechanisms may also exist separately or in combination with the KDEL retention system to localise luminal ER proteins to the ER. As has been mentioned previously it is possible for proteins containing the C-terminal signal to be secreted albeit at a slower rate (Zagouras and Rose 1989), which suggests that the retention system may not be 100% efficient. It is possible however that the folding of these engineered proteins does not allow efficient recognition of the KDEL sequence.

The possibility of calcium ions playing a role in the retention of luminal ER proteins (reticuloplasmins) came from the observation that agents that perturbed the level of cellular calcium caused reticuloplasmins to be secreted (Booth and Koch 1989). A number of possible roles for the involvement of calcium in the sorting of reticuloplasmins have been proposed. One model has been proposed in which relatively high calcium levels promote binding of KDEL-containing proteins to the KDEL receptor in the salvage compartment and relatively low levels of calcium facilitate their release in the ER (Kelly 1990). If this model is correct then the site of action of the calcium-perturbing agents would be the salvage compartment. Another aspect of the KDEL based retention system is the requirement of vesicles containing the reticuloplasmins to return to the ER. Calcium ions have been shown to be necessary for the transfer of material from the ER to the Golgi (Beckers and Balch 1989), therefore it is possible that they are also needed for the retrieval pathway, and that perturbation of cellular calcium may favour secretion of reticuloplasmins. One problem with the above models is that they do not explain how an increase in reticuloplasmin synthesis during stress responses actually decreases their secretion. There has been a suggestion that the level of reticuloplasmins may actually affect the sorting mechanism.

The protein concentration in the ER has been estimated to be as much as 100 mg/ml (Koch 1987). Proteins in other compartments at comparable concentrations do not necessarily exist as freely diffusible monomers or oligomers but aggregate to form a

matrix or a gel. For example, in the sarcoplasmic reticulum calsequestrin is thought to form a matrix stabilised by calcium ions that concentrates the protein in the region of transverse tubules (Messner 1975) . In the bacterial periplasm proteins and proteoglycans form a gel that provides stability and controls diffusion of proteins in that space (Hobot et al 1984). Booth and Koch (1989) suggest that the ER is made up of a gel matrix of reticuloplasmins and that true secretory proteins are part of an additional mobile phase. When vesicles bud from the ER they would preferentially sample the fluid phase and exclude the majority of the immobile reticuloplasmins. Some reticuloplasmins would inevitably escape this bulk sorting and need to be retrieved by a more specific system ie the KDEL system. If this matrix is stabilised by calcium, then calcium perturbing-agents would destabilise the matrix and reticuloplasmins would leave the ER in vesicles in greater amounts than usual. The high concentration of KDEL-containing proteins leaving the ER could saturate the retrieval system and a large proportion of the reticuloplasmins would be secreted along with normal secretory proteins.

1.2 Yeast as an experimental organism

The yeast *Saccharomyces cerevisiae* is a unicellular eukaryote that has been widely used as an experimental eukaryote for a number of reasons. It is amenable to both classical genetic and to molecular genetic techniques using recombinant DNA technology (Struhl 1983). In fact virtually all of the molecular genetic techniques that can be used in bacterial systems can also be applied to *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* has many of the features of higher eukaryotic cells. For example the genetic material is confined to the nucleus in the form of chromatin and organised on many chromosomes each of which has a centromere, two telomeres and multiple sites of DNA replication. The yeast transcription and translation processes have typical eukaryotic features, the proteins are typically eukaryotic and the macromolecular structures resemble those of higher eukaryotes (eg 80S ribosomes). Also the membrane bound organelles in yeast;-RER, Golgi, nucleus, mitochondria and lysosomes-all resemble the organelles of higher eukaryotes.

The biology of yeast involves the fusion of haploid cells of opposite mating types to form diploid zygotes which in appropriate conditions can undergo fairly typical meiosis. Both the haploid and diploid forms of *S. cerevisiae* can be grown vegetatively in defined medium. The amenability to genetic manipulation and the typically eukaryotic features make *S. cerevisiae* useful for studying many of the basic questions in eukaryote biology including ; gene expression, DNA replication, recombination, chromatin structure, the cell cycle, control of cell type and the secretory pathway.

1.2.1 Analysis of the yeast secretory pathway

Stages of the yeast secretory pathway have been defined by the isolation and characterisation of temperature-sensitive mutants in which protein export is blocked at the restrictive temperature. At the restrictive temperature these secretion-defective mutants (*sec* mutants) accumulate organelles and also various precursor forms of the secreted enzyme invertase (Esmon *et al* 1981), the vacuolar enzyme caboxypeptidase Y (CPY) (Stevens *et al* 1982), the mating pheromone α factor (Julius *et al* 1984), and the *S. cerevisiae* killer toxin (Bussey *et al* 1983), which relate to their position in the secretory pathway.

Yeast cells that are temperature sensitive for secretion become dense during incubation at the non-permissive temperature of 37°C due to the accumulation of secretory proteins intracellularly. This property was observed for the original *sec* mutants and used to isolate further secretory mutants by sedimentation of mutagenised cells on a Ludox gradient (Novick *et al* 1980). Colonies derived from these dense cells were screened for conditional growth and conditional secretion of invertase and acid phosphatase. The *sec* mutants that accumulated a large intracellular pool of invertase at 37°C (Class A *sec* mutants) were found to fall into 23 complementation groups. Many of the *sec* mutants secreted their invertase on return to the permissive temperature of 26°C. Electron microscopy (EM) of all of the *sec* mutants (with one exception) revealed the temperature dependent accumulation of membrane-enclosed secretory organelles (see table 1.1). Novick *et al* 1980 described these organelles as being intermediates in the secretory pathway whose soluble contents are destined for secretion by exocytosis and membranes for incorporation into into the plasma membrane by fusion.

A set of 10 of the secretory mutants appeared to accumulate membrane-enclosed vesicles of 80-100 nm diameter which were proposed to represent secretory vesicles before fusion with the plasma membrane. A set of 9 secretory mutants accumulated a more extensive network of ER than seen in wild type cells and a subset of 4 from this group accumulated small 40 nm vesicles as well as ER. Two of the *sec* mutants accumulated structures consisting of two curved membranes with an electron-transparent lumen thought to represent the Golgi complex but termed the Berkeley body (Bb). The *sec* 19 and *sec* 11 mutants could not be placed into any of

Table 1.1 The class A sec mutants

sec mutation	Number of isolates	Organelle accumulated at 37°C
sec1	12	SV
sec2	69	SV
sec3	3	SV
sec4	9	SV
sec5	26	SV
sec6	6	SV
sec7	4	Berkeley bodies
sec8	10	SV
sec9	7	SV
sec10	3	SV
sec11	12	no organelle accumulated
sec12	4	ER
sec13	4	ER
sec14	4	Berkeley bodies
sec15	2	SV
sec16	2	ER
sec17	1	ER, small vesicles
sec18	2	ER, small vesicles
sec19	1	ER, Berkeley bodies, and SV
sec20	1	ER
sec21	1	ER
sec22	4	ER, small vesicles
sec23	1	ER

Twenty three complementation groups have been identified by Novick *et al* (1980). Some of these groups have many representatives but others have only one; this distribution suggests that there are other sec A complementation groups that have not been discovered.

the above groups as *sec* 19 accumulated all three types of organelle and *sec* 11 did not appear to accumulate any organelle under restrictive conditions.

The order of events in the yeast secretory pathway was elucidated by the characterization of haploid double *sec* mutants with respect to the structure of invertase and the amplification of one or more of the secretory organelles mentioned above (Novick *et al* 1981). The morphology of the exaggerated organelles accumulated in these double *sec* mutants at 37°C allowed the order in which the *SEC* gene products are required, to be assessed. By looking at the precursor forms of invertase accumulated the order of events in the processing of invertase could be deduced. The results were consistent with the following model;- secretory proteins enter the ER where initial glycosylation steps take place. Nine or more *SEC* gene products plus energy are required to transfer the secretory proteins to a Golgi-like structure where further glycosylation steps occur. Two or more *SEC* gene products plus energy are required to package the nearly fully glycosylated proteins into secretory vesicles that are then transported to the bud of the cell. Ten or more *SEC* gene products plus energy are then needed for the fusion of these secretory vesicles with the plasma membrane.

1.2.2 Biogenesis and secretion of α factor

Saccharomyces cerevisiae is capable of existing as three cell types;- two haploid cell types designated a and α which can fuse together to form an a/α diploid,-the third cell type. The process of a and α mating is initiated by the reciprocal exchange of diffusible oligopeptide pheromones called a and α respectively (for review see Herskowitz 1986).

The mating pheromone α factor is 13 amino acid residues long, encoded by two genes MFα1 and MFα2 which both encode multiple copies of the α factor peptide. MFα1 has 4 copies of the α factor peptide and MFα2 has two copies. Both genes give rise to large precursor polypeptides. The mature α factor peptides are generated by proteolytic processing of the large precursor polypeptides in the secretory pathway. MFα1 encodes a 165 amino acid precursor polypeptide (pre-pro α factor) which has the following features:- a pre-sequence of 20 amino acids which is the signal

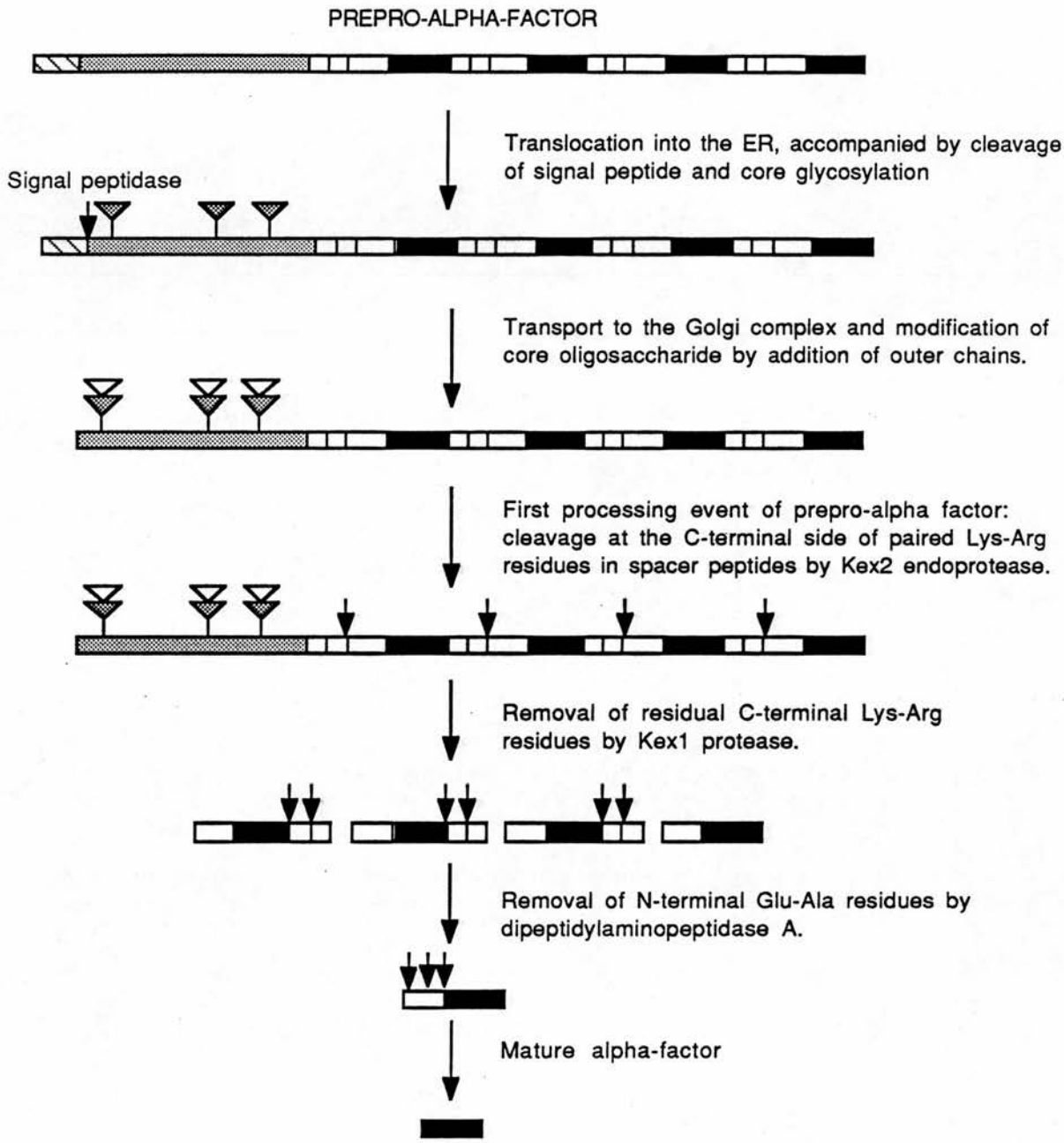
sequence, a 60 amino acid pro-sequence that contains 3 consensus sites for N-linked glycosylation and an N-terminal region which encodes 4 copies of the mature α factor peptide. The 4 repeat sequences are separated by the spacer sequences Lys-Arg-Glu/Asp-Ala-Glu-Ala (see fig. 1.2 and Kurgan and Herskowitz 1982).

The biogenesis of α factor has been assessed with the aid of the *sec* mutants blocked at various stages of the secretory pathway (Julius *et al* 1984). The pre-pro α factor is directed into the secretory pathway by its signal sequence which is removed by signal peptidase (Waters *et al* 1988). The species of pro α factor accumulated in a *sec 18-1* mutant at 37°C (blocked in ER to Golgi transport) has a molecular weight of around 25K which is consistent with the addition of 3 N-linked core oligosaccharides at the consensus positions in the pro region, onto the pro α factor. Limited digestion with endoglycosidase H of the α factor accumulated in *sec18-1* cells at 37°C and treatment of *sec 18-1* cells with tunicamycin (which blocks the synthesis of N-linked glycosyl chains) (Mahoney and Duskin 1979) give results consistent with the addition of three N-linked oligosaccharides to pro α factor (Julius *et al* 1984).

Proteolytic processing of pro α factor is thought to occur during transit through the Golgi apparatus as no proteolytically processed α factor can be detected in a *sec* 18-1 mutant at 37°C. The first cleavage of pro α factor is thought to be carried out by the Kex2 protease at pairs of basic residues in the spacer peptides to release four pheromone repeat units (see fig. 1.1) (Julius *et al* 1984). The Glu/Asp-Ala residues at the amino terminus of each peptide are removed by dipeptidylaminopeptidase-A (Julius et al 1983), and the carboxy terminal Lys-Arg residues are removed sequentially by Kex1 exoprotease (Dmochowska et al 1987, Aschetter and Wolf 1987) (for review see Fuller et al 1988). The proteolytic processing events must be initiated after the position in the secretory pathway at which *SEC7* exerts its effect as material accumulated in a *sec7* mutant at 37°C shows little evidence of being processed. Processing of α factor must be completed before *SEC1* exerts its effect as mature α factor accumulates in a *sec1* mutant at 37°C. The three enzymes responsible for the proteolytic processing of pro α factor have been identified, biochemically characterised and the structural gene for each

Fig1.2 Biosynthesis of the mating pheromone α factor

This diagram shows the post-tranlational processing of the *MFα1* gene product - prepro-a factor. This processing pathway has been elucidated by biochemical and immunocytochemical identification of processing intermediates and by analysis of individual processing enzymes and their genes. See text for further details.


PREPRO-ALPHA-FACTOR
Translocation into the ER, accompanied by cleavage of signal peptide and core glycosylation
Signal peptidase
Transport to the Golgi complex and modification of core oligosaccharide by addition of outer chains.
First processing event of prepro-alpha factor: cleavage at the C-terminal side of paired Lys-Arg residues in spacer peptides by Kex2 endoprotease.
Removal of residual C-terminal Lys-Arg residues by Kex1 protease.
Removal of N-terminal Glu-Ala residues by dipeptidylaminopeptidase A.
Mature alpha-factor
KEY:
pre-sequence
pro-sequence
core glycosylation group
outer chain glycosylation group
alpha-factor repeat unit
proteolytic cleavage site

enzyme has been isolated (see Bussey 1988 for review). The processing of α factor is similar to the processing of prohormones in higher eukaryotes and is the first example of a eukaryotic protein in which all of the intermediates of processing and the enzymes responsible for processing have been identified.

1.2.3 N-linked glycosylation in yeast

In the yeast *Saccharomyces cerevisiae* the early steps in the synthesis of N-linked glycans are identical to those found in animal cells (for review see Kukuruzinska *et al* 1987). The differences occur only in the later processing steps, or in the modifications that the carbohydrate chains undergo in the Golgi. The events that take place in the lumen of the ER are identical to those that take place in higher eukaryotes, ie the transfer of $Glc_3Man_9GlcNAc_2$ from a dolichol pyrophosphate carrier onto selected asparagine residues followed by the stepwise removal of 3 glucose residues and 1 mannose residue (see fig. 1.1). Once these processing events have taken place the glycoproteins are transported in an energy-dependent manner from the ER to the Golgi presumably via specific vesicles. In the Golgi the processing of yeast oligosaccharides diverges from that of animal cells. Outer chain modifications to N-linked carbohydrates still proceed in sequential fashion in yeast, but unlike complex-type oligosaccharides in animal cells the modifications are composed principally of mannoses added in specific linkages by different mannosyl transferases. These mannosyl transferases function in the Golgi apparatus to add mannose residues to core oligosaccharide units in α1-6, α1-2 and α1-3 carbon linkages, where the attachment of mannobiose phosphate in diester linkages also occurs (for review see Ballou 1982).

Mutations that interfere directly with the addition of outer-chain mannose residues do not necessarily prevent subsequent processing events in the secretory pathway, therefore intermediates in the processing of oligosaccharides are difficult to identify using mutants of this type. The use of *sec7* mutant cells to accumulate glycoprotein intermediates in transit allows identification of reactions that precede and follow this Golgi-specific block (Franzusoff and Schekman 1990). In *sec7* cells at the restrictive temperature of 37°C there were no α1-3 mannose linkages detectable in glycoproteins but α1-2 and α1-3 mannose linkages could be detected. Thus it appears that α1-2 and α1-6 linkages occur in a separate compartment or

compartments in the Golgi apparatus before that in which the α1-3 linkages occur. These experiments suggest that the yeast Golgi apparatus has at least 2 functionally seperate compartments which require the Sec7p for transport between the two.

In mammalian cells, one way in which proteins are thought to be targetted to lysosomes is by the recognition of a mannose-6-phosphate marker on their N-linked oligosaccharides (Kornfeld *et al* 1986). Alternative targetting signals must also exist in mammalian cells (Owada and Neufeld 1982, Wakeed *et al* 1982 and 1988). In yeast, inhibition of glycosylation does not prevent targetting to the vacuole (analogous to lysosome) (Hasilik and Tanner1978, Schwaiger *et al* 1982), thus targetting of proteins to the yeast vacuole is not dependent on modifications to their oligosaccharides. Specific sequences in yeast CPY appear to direct it to the vacuole (Johnson *et al* 1987).

1.2.4 Retention of soluble ER proteins in yeast.

As has been discussed in earlier sections the powerful genetics of *Saccharomyces cerevisiae* has been used to identify processes taking place in the yeast secretory pathway and the gene products involved at various stages of the secretory pathway. Many of the processes taking place are analogous to those occurring in higher eukaryotes. The first indication that yeast may have a similar system to that of higher eukaryotes for the sorting of resident ER proteins came from the fact that yeast BiP contained the C-terminal tetrapeptide HDEL which is a similar motif to the KDEL sequence (Pelham *et al* 1988). Yeast PDI also has this HDEL motif at the C-terminus (Dr. R.Freedman personal communication). Antibodies to the HDEL sequence also recognise other yeast proteins (Hardwick *et al* 1990).

Pelham *et al* (1988) showed that an invertase-HDEL fusion protein was retained within yeast cells whereas an invertase-KDEL fusion protein was not efficiently retained. Thus it appears that HDEL is the ER retention signal in yeast and not KDEL. The yeast HDEL sequence, as in mammalian cells, is thought to act as a recycling signal (Pelham et al 1988):- Yeast glycoproteins receive the same oligosaccharide chains in the ER as animal cell glycoproteins. Some are then modified by the addition of 100 or more mannose residues with specific linkages. This outer-chain modification is considered a Golgi function as a *sec18* mutant at the restrictive

temperature of 37°C blocks this modification. The invertase fusion protein bearing the HDEL signal, and at least one endogenous ER protein is subject to this outer-chain modification which suggests that such proteins can reach the Golgi and still be retrieved. The outer-chain glycosylation occurs more slowly with HDEL-tagged proteins than with similar proteins lacking the HDEL sequence, thus exposure of ER proteins to the outer-chain modifying enzymes is probably a rare event and as with higher eukaryotes retrieval may usually be from a pre-Golgi or very early Golgi compartment.

Recent experiments in which pre-pro α factor was tagged at its C-terminus with the HDEL sequence (Dean and Pelham 1990) show that fusion protein accumulates intracellularly in a Sec18-dependent manner with ER and Golgi-specific modifications in the ER as shown by subcellular fractionation. The Golgi-specific modifications to the α factor construct consist of α1-6 mannose linkages but no α1-3 linkages were detectable. The addition of α1-6 linkages has been shown to be an early Golgi event and addition of α1-3 linkages to be a later event (Franzusoff and Schekman 1989) which suggests that retrieval is from an early Golgi compartment. The yeast retention system appears to be saturable as overexpression of the α factor HDEL fusion proteins results in the secretion of BiP which is not normally secreted (Dean and Pelham 1990).

1.2.5 The yeast HDEL receptor

Powerful selection and screening methods have been developed to select a number of mutants defective in retaining HDEL-containing proteins (Pelham *et al* 1988). These mutants are named *erd* mutants for ER retension defective and analysis of the mutants defines two genes- *ERD1* and *ERD2*. The function of the *ERD1* gene product is not clearly understood but it has been suggested that it is required for the correct binding of the HDEL receptor to its ligands (Hardwick *et al* 1990). The *ERD2* gene product appears to be the putative HDEL receptor itself (Semenza *et al* 1990, Lewis *et al* 1990). The *ERD2* gene encodes a 26K integral membrane protein whose abundance determines the efficiency and capacity of the retention system. Also the cloning of the *Kluyveromyces lactis ERD2* gene into *Saccharomyces cerevisiae* changes the specificity of retention of proteins in *S. cerevisiae* to that found in *K. lactis*, ie *S. cerevisiae* will retain proteins with either HDEL or DDEL at the

C-terminus, as does *K. lactis.* Proteins with DDEL at the C-terminus are not normally retained in *S. cerevisiae.* The *ERD2* gene product is also essential for growth, which suggests it may have a function other than that of receptor. It has been suggested that the *ERD2* gene product may be needed to form the recycling vesicles and, if absent, soluble or membrane proteins involved in vesicular trafficking could not return to the ER and reduce the secretory pathway to a trickle. It is interesting to note that the putative KDEL receptor in mammalian cells identified by Vaux *et al* (1990) and the putative HDEL receptor in yeast have very different molecular weights.

1.2.6 The Kex2 protease of *Saccharomyces cerevisiae*

The Kex2 protease (Kex2p) is an endopeptidase that cleaves at the carboxyl side of Lys-Arg or Arg-Arg residues (for review see Fuller 1988). It is required for the maturation of *Saccharomyces cerevisiae* killer toxin (Bostian *et al* 1984) and the mating pheromone α factor (Julius *et al* 1984). The predicted amino acid sequence of the Kex2p contains a domain homologous to the subtilisin class of serine proteases (Mizuno *et al* 1988), a serine/threonine-rich domain thought to be a site of extensive O-linked glycosylation (Sudhot *et al* 1985) and a transmembrane domain that is thought to anchor it in the membrane.

Kex2p is present in very low amounts in yeast as is the case for many membrane proteins but studies in which Kex2p is overexpressed reveal that, Kex2p is membrane bound but solubilised by detergents, is able to cleave peptide substrates at Arg-Arg or Lys-Arg sites and is inhibited by EDTA and EGTA but is fully reactivated by calcium ions (Fuller et al 1989). Also Kex2p can be labelled specifically with ^{125}I labelled Tyr-Ala-Lys-Arg chloromethylketone. Mutant enzymes lacking as many as 200 C-terminal amino acid residues can still be specifically labelled, and retained calcium dependent protease activity (Fuller et al 1989).

The subcellular location of Kex2p has been suggested to be the Golgi for a number of reasons: The processing of pre-pro α factor and pre-pro killer toxin by Kex2p occurs at a kinetically late step in the secretory pathway (Julius et al 1984, Bussey *et al* 1983). No significant levels of Kex2p have been found in enriched fractions of

secretory vesicles or at the surface of intact cells and therefore it is likely that the enzyme functions within the Golgi complex. Kex2 processing is now thought to occur in late Golgi after the *sec7* block and after α1-2 and α1-6 mannose addition to oligosaccharides (Franzusoff and Schekman 1989, Dean and Pelham 1990). It has been reported that immunofluorescence microscopy detects Kex2p in the same subcellular location as Sec7p, in structures that may represent Golgi apparatus (cited in Franzusoff and Schekman 1989 as unpublished data).

1.2.7 The killer toxin of *Kluyveromyces lactis*

Killer strains of the yeast *Kluyveromyces lactis* secrete a protein toxin which inhibits the growth of sensitive yeasts. The production of the toxin is dependent on the presence of two linear double stranded DNA plasmids, k1 and k2 of sizes 8.9kb and 13.4kb respectively (Gunge *et al* 1981, Wesolowska *et al* 1982). The linear plasmids can be transferred to *Saccharomyces cerevisiae* although only to ρ^0 strains as the linear plasmids are not stably maintained in strains containing mitochondrial DNA (ρ^0 strains of yeast lack mitochondrial DNA and are *petite* mutants). Active toxin indistinguishable from that secreted from *Kluyveromyces lactis* is secreted from *Saccharomyces cerevisiae* strains harbouring the linear plasmids. Yeast cells harbouring the linear plasmids are not sensitive to the toxin as the plasmids also confer immunity upon the cells containing them. Both k1 and k2 have been sequenced and have been shown to have 4 and 10 open reading frames (ORFs) respectively. Functions have been assigned to at least 6 of the products of the ORFs (for review see Stark *et al* 1990). The plasmid k2 is thought to function in the maintenance of both of the linear plasmids in the cell by encoding transcription factors etc, as it is possible to find cells with k2 alone but not with k1 alone. The plasmid k1 has been shown to encode the structural genes for the toxin (Stark and Boyd 1986) (see fig. 1.3).

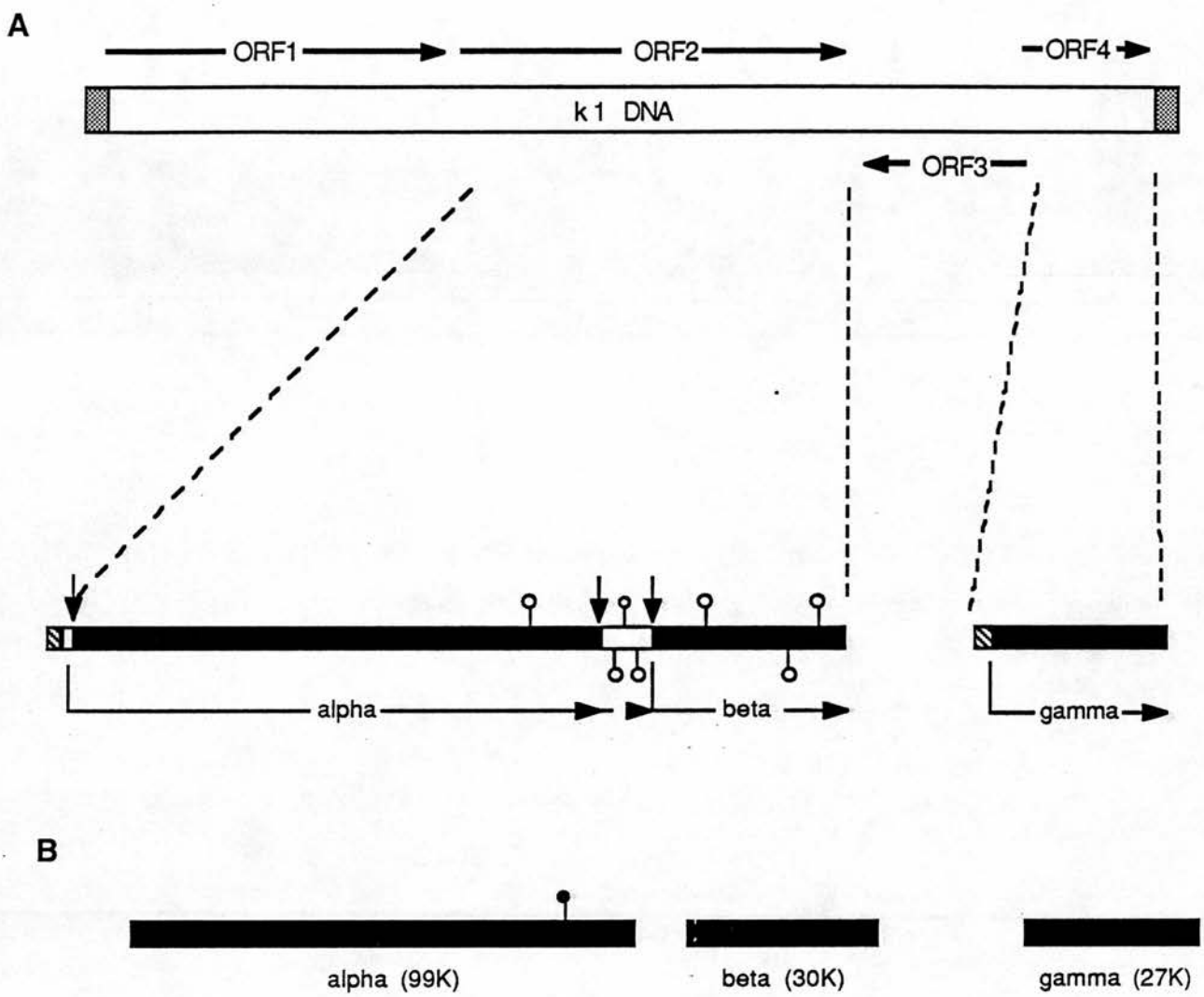
1.2.8 Structure of the toxin

The toxin secreted from linear plasmid-containing strains of yeast consists of 3 subunits, α, β and γ with molecular weights of 99K, 30K and 27K respectively (Stark and Boyd 1986). The α subunit is glycosylated with a single core N-linked oligosaccharide but neither of the smaller subunits appears to be glycosylated. The

Fig. 1.3 **The linear plasmid k1 and the toxin polypeptides that it encodes**

(A) The linear plasmid k1 has four open reading frames (ORF's). ORF2 and ORF4 encode the toxin polypeptides. ORF2 encodes a 130 K primary translation product (the αβ precursor) which is processed in the secretory pathway to give rise to the mature α and β subunits. The αβ precursor polypeptide contains 3 potential Kex2 cleavage sites. It is known that the N-terminus of the mature α subunit starts immediately after a potential Kex2 cleavage site as does the N-terminus of the β subunit. The exact C-terminus of the α subunit is not known, there are two possibilities:- It is possible that the cleavage at the potential Kex2 site that creates the N-terminus of the β subunit creates the C-terminus of the a subunit. If however all of the potential Kex2 cleavage sites are cleaved a small 'spacer peptide' will be excised from the precursor to create the C-terminus of the α subunit. Maturation of the ORF4 primary translation product only involves the removal of the signal sequence by signal peptidase.

(B) The mature toxin polypeptides are shown. However there is some doubt about the α subunit due to not knowing its exact C-terminus. I have assumed that all the potential Kex2 sites have been cleaved and thus there is only one possible site for N-linked glycosylation. However if the 'spacer peptide' is not excised, as may be the case there are four potenial sites for N-linked glycosylation each which could be the actual site of glycosylation. The β subunit is not glycosylated even though it has potential sites for N-glycosylation, this may be due to the unusually hydrophobic nature of the β subunit. The γ subunit contains no potential sites for N-glycosylation.


A
ORF1
ORF2
ORF4
k1 DNA
ORF3
alpha
beta
gamma
B
alpha (99K)
beta (30K)
gamma (27K)
KEY
Signal peptide
Consensus site for N-linked glycosylation
Most probable N-linked glycosylation site.
Kex2 cleavage sequences

structure of the toxin is maintained by disulphide bonds:- The β and γ subunits are thought to be covalently attached by disulphide bonds to one another and the α subunit is thought to contain at least one intramolecular disulphide bridge, as implied by analysis on non-reducing SDS-PAGE (Tokunaga *et al* 1987). A stoichiometry of 1:1:1 for the subunits is likely but this point still needs to be clarified.

The α and β subunits of the toxin are produced as a large precursor (the αβ precursor) and proteolytically processed to give rise to the 2 mature subunits. The *S. cerevisiae* Kex2 protease has been implicated in this processing as amino acid sequencing of the mature subunits reveals that the N-terminal amino acids of the mature subunits are those that would be predicted if cleavage of the αβ precursor were to take place at the C-terminal side of Lys-Arg residues (Stark *et al* 1986).

1.2.9 Toxin assembly.

The *K.lactis* toxin is structurally complex and therefore it is likely that specific assembly steps are involved in its biogenesis. Evidence to support this is the fact that the γ subunit fails to be secreted in the absence of αβ production even though the leader peptide is functional in facilitating translocation into the lumen of the ER of the γ subunit (Tokunaga *et al* 1989) and can be used to direct secretion of heterologous proteins (Tokunaga *et al* 1988). However if the *S.cerevisiae* pro α factor sequence is inserted in frame between the γ subunit leader peptide and the coding sequence of the mature polypeptide the γ subunit is secreted (Tokunaga et *al* 1989). This is interesting as the γ subunit is the only example of a secreted yeast protein that is neither glycosylated nor cleaved from a glycosylated precursor. Secretion of the γ subunit does not occur unless it associates with the other toxin subunits or α factor pro-sequence is added. In either case the γ subunit is being associated with a glycosylated entity. There is some confusion as to the mode of action of the toxin but the γ subunit has been implicated as being the actual toxic subunit (for review of possible modes of action see Stark *et al* 1990).

The *K.lactis* killer toxin has a number of features that make it attractive and

interesting for the study of biogenesis of proteins in the yeast secretory pathway. It is the only example of a multi-subunit yeast protein that is assembled from the products of multiple transcripts and the mature subunits can be identified from one another by mobility on SDS PAGE. Two of the subunits (β and γ) are linked covalently by disulphide bonds, therefore it should be possible to monitor the assembly of the toxin by direct immunoprecipitation. One of the subunits (α) is glycosylated and the precursor has other consensus sites for glycosylation. Also proteolytic processing events should be easy to monitor because of large size differences in the precursor and mature polypeptides.

1.3 Outline of project.

At the outset, the aim of my project was to examine the biogenesis of secretory proteins in yeast in order to further our knowledge of processes that take place in the secretory pathway. The assembly and processing of the *K.lactis* killer toxin was examined, since it is an unusual yeast protein (in that it is a multi-subunit secreted protein), and may also provide information that can be related to the analogous assembly of multi subunit secreted proteins in higher eukaryotic cells. As the project progressed, the question as to whether the order of processing events was important in the maturation of proteins was addressed. This was done by attempting to re-locate a Golgi processing enzyme (Kex2 endoprotease) to the ER by the addition of specific ER retention signals, and then examining the maturation of yeast secretory proteins (i.e. mating pheromone α factor and *K.lactis* killer toxin) when processed prematurely (if at all) by this abnormally located enzyme. The attempt to relocate the Kex2 protease activity to the ER was also of interest as it provided insight into the retention of luminal ER proteins in *S.cervisiae*.

Chapter two.

Materials and Methods.

2.0 Chemicals

All chemicals were obtained from BDH Chemicals, Sigma Chemical Co, or Fisons Ltd, Loughborough. The synthetic peptide substrate for the Kex2 assays (b-QRR MCA) was obtained from Peptide Intitute Inc, Japan

2.1 Radiochemicals

[α-^{35}S]-dATP (500 Ci/mmol) was obtained from New England Nuclear. [^{35}S] methionine (1115 Ci/mmol) and [α-^{32}P]-dATP (>3000 Ci/mmol) was from Amersham International plc.

2.2 Enzymes

All DNA modification enzymes including restriction endonucleases, phage T4 DNA ligase and the Klencw fragment of DNA polymerase I were obtained from Bethesda Research Laboratories (BRL). Zymolyase 100T was from the Seikagaku Kogyo Co, Japan. Lysozyme was from Sigma Chemical Co.

2.3 Bacterial and yeast strains

The strains of *E. coli* and *S. cerevisiae* are listed in Table 2.1, with their relevant properties. Derivatives of these strains are not listed but are mentioned in the relevant Chapters

2.4 Media

In general bacterial cultures were grown in complete medium (Luria broth, LB) containing 0.1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, and 0.5% (w/v) NaCl. When appropriate the antibiotics ampicillin (100 μg/ml) or

Table 2.1 Bacterial and yeast strains

Bacterial	Genotype	Source
E.coli NM522	Δ (*lac-pro*AB), *his*Δ5, (r_K^-, m_K^+) *thi*, *sup*E, F' *pro*AB *lacI*^{Q}ZΔM15.	Gough and Murray (1983)
E.coli pop2136	not available	Kusters *et al* (1989)
Yeast		
S.cerevisiae MRY101	*MAT*a; *gal2*; *sec*18-1; ρ^0; [k1, k2]	Dr. M. Romanos
S.cerevisiae MRY102	*MAT*a; *gal2*; *sec*14-3; ρ^0; [k1, k2]	Dr. M. Romanos
S.cerevisiae MRY103	*MAT*a; *gal2*; *sec*1-1; ρ^0; [k1, k2]	Dr. M. Romanos
S.cerevisiae LL20	*MAT*α; *leu2*-3,112; *his*3-11,15	Dr. C. Hadfield
S.cerevisiae RC631	*MAT*a; *sst2*; *rme*; *ade2*; *ura*1; *his*6; *met*1; *can*1; *cyh*2; GAL.	Chan and Otte (1982)
S.cerevisiae ABYS106	*MAT*?; *pra*1-1; *prb*1-1; *prc*1-1; *cps*1-3; *ura*3; *leu2*.	Dr. A. Boyd
S.cerevisiae JRY188	*MAT*α; *leu2*-3,112; *ura*3-52; *trp*1; *his*4; *sir*3; *rme*.	Brake *et al* (1984)
S.cerevisiae F102-2	*MAT*a; *leu2*-3,102; *his*4-519; *can*1; [k1, k2]	Dr. N. Gunge
S.cerevisiae PWYS1	F102-2 (*kex2::LEU2*)	This study
S.cerevisiae PWYS2	JRY188 (*kex2::LEU2*)	This study
S.cerevisiae PWYS3	JRY188 (*kex2::URA3*)	This study
S.cerevisiae PWYS4	F102-2 (*kex2::LEU2, trp1::λ*)	This study

kanamycin (80 μg/ml) were added to the media. When solid medium was required, 2% (w/v) Bacto agar was added to the above media.

Yeast cultures were grown in complete medium (YPD), containing 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone and 2% (w/v) glucose; or minimal (selective) medium (SD), 0.67% (w/v) Bacto yeast nitrogen base without amino acids and 2% (w/v) glucose. When required the following nutrients were added: histidine (20 μg/ml), leucine (30 μg/ml), tryptophan (20 μg/ml) and uracil (20 μg/ml). SD supplemented with 1% Casein hydrolysate (which lacks uracil and tryptophan) was used in a number of experiments. Where appropriate the carbon source used was 2% (w/v) galactose (YPG, SG) instead of glucose (YPD, SD). When solid medium was required 2% (w/v) Bacto agar was added to the above media.

2.5 General manipulations of DNA

All standard techniques including restriction endonuclease cleavage, ligation, extraction with phenol, and precipitation in ethanol were carried out as described by Maniatis et al., (1982). Gel electrophoresis for visualising DNA fragments was routinely carried out using 0.8% (w/v) agarose gels; the size of the DNA fragments was estimated by comparison with a Pst I digest of phage λ DNA.

2.6 Transformation of bacterial and yeast cells

DNA was transformed into bacterial cells previously treated with $CaCl_2$ as described by Maniatis et al (1982). Yeast cells were made competent for transformation with LiOAc following the protocol of Ito et al (1983).

2.7 Small scale preparation of genomic DNA from yeast

The yeast strain was grown in YPD overnight to a high density (stationary phase of growth). A total of 4.5 ml (3 x 1.5 ml) of culture was harvested in an Eppendorf tube by centrifugation in a microfuge. The cell pellet was resuspended in 800 μl of

BME buffer (0.9 M sorbitol, 0.05 M Na_2HPO_4, containing 1 µl of β-mercaptoethanol per ml of buffer). 25 µl of zymolyase (10 mg/ml) was added to the resuspended pellet and mixed by inversion. Incubation was for 30-45 min at 37°C until spheroplasts could be seen to be formed. The spheroplasted cells were pelleted by centrifugation at low speed in a microfuge and resuspended in 100 µl of 1 M sorbitol. 800 µl of lysis buffer (0.1M Tris HCl pH 9.7, 50 mM EDTA pH 8.5, 0.5% (w/v) SDS) was added to the spheroplasts and incubated at 70°C for 20 min. After the 20 min incubation, 200 µl of 5 M KOAc was added, mixed and left on ice for 45 min. The tubes were centrifuged in the microfuge at high speed for 10 sec and the supernatant was carefully transferred to a clean Eppendorf tube without transferring any of the precipitate. 0.55 ml of isopropanol was added to the supernatant and left at room temperature for 5 min to precipitate the DNA. The DNA was pelleted by centrifugation at high speed in a microfuge for 10 minutes. The DNA pellet was washed once in 70% ethanol, dried and resuspended in 20 µl of TE (it was necessary to heat the pellet at 65°C in order to resuspend it). 10 µl of this DNA was digested and ran on 0.8% agarose gels for Southern analysis.

2.8 Southern hybridisation

Transfer of DNA from agarose gels to nitrocellulose filters, and the subsequent hybridisation of α-[^{32}P] radiolabelled probes to the filter,were carried out as described by Maniatis (1982) using a method based on that of Southern (1975).

2.9 Preparation of radiolabelled DNA probes

The DNA fragment to be labelled was purified from an agarose gel (Maniatis et al 1982). Approximately 25 ng of purified DNA was labelled following the method of Feinberg and Vogelstein (1983): DNA synthesis was primed on denatured DNA by random hexadeoxynucleotide (obtained from Pharmacia) in the presence of the Klenow fragment of *E. coli* DNA polymerase I and α-[^{32}P]-dATP.

2.10 DNA sequence analysis : Preparation of single stranded M13 DNA

A single M13 plaque was used to infect 20 µl of exponentially growing *E. coli* NM522 cells in a 30 ml polypropylene tube. After 5 minutes at room temperature, 1.5 ml of 2 x TY (1.6% (w/v) Bacto tryptone, 1% (w/v) Bacto yeast extract, 0.5% (w/v) NaCl) growth medium was added and the cultures were incubated at 37°C overnight (16 hrs). During the incubation the cultures were shaken vigorously to provide adequate aeration. The cultures were transferred to 1.5 ml Eppendorf tubes and the cells were harvested by high speed centrifugation in a microfuge. Supernatants were decanted into fresh eppendorf tubes and single-stranded DNA was prepared from these stocks. Bacterophage were precipitated by addition of 200 µl of 20% (w/v) PEG 6000, 2.5 M NaCl solution to 900 µl of supernatant. After 60 minutes at room temperature the phage pellet was collected by high speed centrifugation in a microfuge and the pellet was resuspended in 200 µl TE buffer. This was extracted twice by the addition of an equal volume of a 1:1 phenol: chloroform solution, after which the DNA was precipitated out of the aqueous phase in the presence of 1/10 volume of 3 M NaOAc pH 5.5 and 2 volumes of ethanol at -20°C. The precipitated DNA was collected by centrifugation at high speed in a microfuge for 10 minutes and the pellet was resuspended in 20µl of TE buffer. 5 µl of this DNA was used as template in a typical sequencing experiment.

The single stranded M13 DNA was sequenced using the dideoxy chain termination method of Sanger et al (1977) in the presence of [α-^{35}S]-dATP (Williams *et al* 1986). The sequencing reactions were performed as described in the BRL " M13 cloning/dideoxy sequencing instruction manual " using a BRL sequencing kit. The sequencing reactions were electrophoresed on a 6% acrylamide gel (0.4 mm thickness) containing 8 M urea.

2.11 In situ lysis gels to detect linear DNA plasmids k1 and k2.

The yeast cells to be checked for the presence of the linear plasmids were grown overnight in the appropriate medium (either YPD or supplemented SD if selective growth required). 1 ml of each of the cultures to be tested were harvested by centrifugation in a microfuge and the cell pellet was washed with 1 ml of SE (1 M sorbitol, 25mM EDTA). The cells were harvested again and resuspended in 50 µl SED (SE containing 50 mM DTT) and then 5 µl of zymolyase (5 mg/ml) was added. The cells were incubated at 37°C or until spheroplasts were formed. To check for the presence of the linear plasmids the spheroplasts were lysed in an agarose gel (in situ) to release their DNA content. The gel was poured using two sets of slot formers taped back to back so that the two rows of slots were about 3 mm apart. The back slot former was taped so that it created a single large slot. The leading wells were loaded with the spheroplasts and the trailing well with the lysis buffer (1 ml lysis buffer= 0.9 ml 1% SDS, 0.1 ml gel loading buffer, 10 ml RNase A (10 µg/ml)). The samples were electrophoresed overnight at 15 V. As the lysis buffer runs through the spheroplasts it gently lyses them releasing the DNA content. The gels were observed under UV illumination after staining in ethidium bromide solution (Maniatis *et al* 1982)

2.12 Making radiolabelled yeast extracts

For the labelling of the *sec* mutant strains of yeast posessing the linear plasmids (MRY101, MRY102 and MRY103) 5 mls of the appropriate strain was grown at 26°C in labelling medium (SD + HUWLK, 5% (v/v) glycerol, 1% (w/v) BSA). When the cells were at a density of approximately 2 x 10^7 cells/ml they were moved to 37°C for 10 min to introduce the *sec* block. Once the *sec* block had been introduced, 5 µl (50 µCi) of ^{35}S-methionine was added to the culture and the cells were placed back at 37°C for 90 mins. For the last 2 min of labelling 10 mM sodium azide was added to the culture and the cells were placed on ice. The cells were harvested in an Eppendorf tube by centrifugation in a microfuge (5 x 1 ml). and the supernatants were discarded. The cell pellet was washed twice in water and then resuspended in 50 µl of 1% SDS. 0.3 g of glass beads were added to the Eppendorf

tube and the tube was vortexed (5 x 30 sec) to break the cells. 500 µl of lysis solution (0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, in 1 x PBS) was added and the tube vortexed again. The mixture was placed in a boiling water bath for 5 mins and then a hole was punctured in the bottom of the Eppendorf with a needle. The Eppendorf was placed in a small Falcon tube and the extract was forced through the hole in the Eppendorf tube into the Falcon tube by centrifugation (1000 rpm for 2 mins in Beckman J2-21 rotor). 50 µl of 10 x protease inhibitors (200 µl of 200mM PMSF prepared in isopropanol, 20 µl of 1 mg/ml pepstatin prepared in methanol , 20 µl of 1 mg/ml antipain prepared in water, 20 µl of 1 mg/ml leupeptin prepared in 3:1 methanol/DMSO, 20 µl of 1 mg/ml chymostatin prepared in 3:1 methanol/DMSO made up to 1 ml with water) was added to the extract in the Falcon tube. The extract was transferred to an Eppendorf tube and centrifuged for 10 minutes at high speed to get rid of unbroken cells and cell debris. The supernatant was transferred to a clean Eppendorf tube. Extracts prepared in this way were used in the immunoprecipitation experiments.

2.13 Immunoprecipitation of labelled proteins.

50 µl of the labelled extract was placed into an Eppendorf tube containing 150 µl of lysis solution (see above). 5 µl of serum (either immune or pre immune) was added to the tube and the tube was placed on a rotating wheel overnight in the 4°C cold room. The next morning 30 µl of Pansorbin fixed *Staphylococcus aureus* (Staph A) cells (or 30 µl of protein A-sepharose) was added to the tube and incubated at 4°C on the rotating wheel for a further 1 hr. The Staph A cells (or protein A-sepharose) were pelleted in the microfuge and washed twice in HEN (100 mM Hepes NaOH pH 7.9, 10 mM EDTA, 1 mM NaCl, 1% (v/v) NP40), three times in LIT (0.5 M LiCl, 0.1 M Tris HCl pH 8.8, 0.1% (w/v) SDS, 1% (v/v) NP40) and then once again in HEN (30 minutes for each wash on the rotating wheel at room temperature). The final pellet was resuspended in 30 µl of protein sample buffer and boiled for 3 minutes. The immunoprecipitates were analysed on 10% polyacrylamide gels.

2.14 Induction of transformed NM522 cells to produce protein A fusion proteins

E. coli strain NM522 transformed with pKPRA (or plasmid derived from pKPRA) was grown in 100 ml of selective medium (LB + Kanamycin) at 37°C to a density of A_{600} = 0.3-0.5. When the cells had reached this density IPTG was added to a concentration of 0.25 mM to induce cells to produce fusion protein from the lac promoter. Induction was for 4 hrs at 37°C after which the cells were harvested and inclusion bodies were prepared.

2.15 Induction of transformed pop2136 cells to produce β-galactosidase fusion proteins.

E. coli strain pop2136 transformed with a pEX plasmid (or a plasmid derived from the pEX plasmids) was grown in 100 mls of selective medium (LB + ampicillin) at 30°C to a density of A_{600} = 0.4. When the cells had reached this density they were moved to a 42°C shaking incubator to induce the cells to produce the fusion protein. Induction was for 4 hrs at 42°C after which the cells were harvested and inclusion bodies were prepared.

2.16 Preparation of inclusion bodies from *E. coli*

The purification of inclusion bodies from NM522 and pop2136 was the same even though the method of induction was different. Although the fusion proteins in this thesis all produced inclusion bodies when cells were induced for their expression, it has to be noted that some fusion proteins do not appear to form inclusion bodies but are apparently soluble in the cell, in which case it is of no use to purify inclusion bodies. The inclusion bodies were purified by the following method: 100 ml of induced *E. coli* culture was harvested by centrifugation and washed in 50 ml of 0.1 M Tris HCl, pH 7.5. The cells were lysed by the addition of 1 mg/ml lysozyme, incubation on ice for 20 mins and then sonication for 5 x 30 sec (between each sonication the cells were placed on ice for 1 min to prevent excess warming). Cell

debris was removed by centrifugation at 1000 rpm (in a Beckman JA-20 rotor) for 5 min. The supernatant was poured into a clean tube and the inclusion bodies were pelleted by centrifugation at 18000 rpm (in a Beckman JA-20 rotor). The inclusion bodies were resuspended in 0.5 ml 100 mM Tris HCl, pH 8, 150 mM NaCl, 6 mM β-mercaptoethanol, 1% (w/v) SDS, 1% (v/v) Triton X-100. The pellets were usually difficult to dissolve and therefore needed to be heated to 65°C to aid solubilisation. 0.5 ml of protein sample buffer was added to the preparation; samples were boiled for 5 min before analysis on polyacrylamide gels.

2.17 Preparation of concentrated supernatants and crude microsomes from yeast.

The yeast strains were each grown overnight in 100 ml of their selective media (SD + Casein amino acids) to stationary phase of growth. The cells were pelleted by centrifugation at 1500g for 10 minutes and the supernatants were removed. The supernatants from each of the strains were concentrated from 100 ml to approximately 1.5 ml by filtration under pressure through an Amicon ultrafiltration cell (YM 2 filter). The crude microsome preparation was as follows:- The cell pellet was resuspended in 5 mls of 10mM Tris.HCl pH 7.3, 1mM EDTA, 150mM NaCl. An equal volume of glass beads was added to the cell suspension and vortexed for 5 x 30 seconds to lyse the cells, placing on ice between each vortexing for 1 min. A hole was punctured in the bottom of the tube containing the lysed cell/glass bead suspension and this tube was placed into a larger centrifuge tube. The lysed cell suspension was spun through the hole and into the larger centrifuge tube at low speed (300 g) for 5 minutes. The supernatant in the larger centrifuge was transferred to a clean centrifuge tube, leaving the pellet of cell debris and unlysed cells behind. The supernatant was centrifuged at 38000g for 20 minutes to pellet the microsomes. The supernatant from this spin was discarded and the microsome pellet was resuspended in 100 µl of 50mM potassium acetate pH 5.0 , 1% (w/v) Brij 58.

2.18 Gel electrophoresis of proteins.

Separation of proteins was by electrophoresis through polyacrylamide gels following the basic procedures and buffer system of Laemmli (1970). The constitution of the various buffers and solutions is given in Table 2.2. Electrophoresis was usually carried out on the Hoeffer "tall mighty small" apparatus although larger slab gels 18 x 14 cm were also used in some case.10% (w/v) acrylamide separating gels with a 5% (w/v) stacking gel were routinely used.

Protein bands on gels were visualised by staining with Coomassie Brilliant Blue:- The gel was first immersed in fixing solution (10% (v/v) acetic acid, 20% (v/v) methanol) for 30 minutes with gentle agitation. Protein bands were then stained with 0.25% (w/v) Coomassie Brilliant Blue dissolved in 50% (v/v) methanol, 7.5% (v/v) acetic acid for 10 minutes. Gels were destained by agitation in 10% (v/v) methanol, 7% (v/v) acetic acid.

For fluorography, radioactive gels were fixed in 10% acetic acid, 20% (v/v) methanol, washed extensively in water and then placed in 10 volumes of the fluor (1 M sodium salicylate) for 30 mins. The gels were then dried onto a piece of Whatman 3 MM chromatography paper, and exposed at -70^{o}C to X-ray film (Amersham hyperfilm).

2.19 Transfer of proteins onto nitrocellulose

Proteins separated on acrylamide gels were transferred onto nitrocellulose membranes using a semi dry blotting procedure (The apparatus used was an LKB semi dry blotter). The procedure used was as follows: Six pieces of 3 MM paper and one piece of nitrocellulose were cut to the same size as the gel to be transferred. Two pieces of 3 MM paper were soaked in anode buffer 1 (0.3 M Tris, 20% (v/v) methanol, 0.1% (w/v) SDS, pH 10.4) and placed onto the anode plate. A piece of 3 MM paper soaked in anode buffer 2 (25 mM Tris, 20% (v/v) methanol, 0.1% (w/v) SDS, pH 10.4) was then placed on top of the two pieces of 3MM soaked in

Table 2.2 Solutions and buffers used in polyacrylamide gel electrophoresis

A. Separating gel buffer

0.75 M Tris.HCl pH8.8, 0.2% (w/v) SDS

B. Stacking gel buffer

0.25 M Tris.HCl pH6.8, 0.2% (w/v) SDS

C. Acrylamide solution

44% (w/v) acrylamide, 0.8% (w/v) N;N'-methylene-bis-acry--lamide (bis)

D. Electrophoresis buffer

0.125 M Tris, 0.192 M glycine, 0.1% (w/v) SDS

(gives pH8.3 without adjustment)

E. Sample buffer

0.0625 M Tris.HCl pH6.8, 20% (w/v) glycerol, 4% (w/v) SDS, 5% (w/v) β-mercaptoethanol

anode buffer 1, followed by a piece of nitrocellulose soaked in anode buffer 2. The gel, soaked in cathode buffer (25 mM Tris, 20% (v/v) methanol, 0.1% (w/v) SDS, 40 mM 6-amino-n-hexanoic acid, pH 9.4) was placed on top of the nitrocellulose followed by three pieces of 3 MM paper soaked in cathode buffer. The cathode plate was placed on top of the stack, sandwiching the stack together. Transfer was at 0.8 mA per cm^2 of gel area for 1 hr (ie the current used was dependent on the size of the gel). Care was taken when forming the stack, not to trap air bubbles between the layers as this may have interfered with transfer. After the proteins had been transferred for an hour the assembly was taken apart and the nitrocellulose was treated for the next stage (ie treated for Western analysis or used as an affinity strip).

2.20 Purification of fusion proteins from polyacrylamide gels.

A double thickness (2 mm) 10% polyacrylamide gel (large format) was loaded with the preparation of inclusion bodies containing the desired fusion protein (in this case the protein A-Kex2 fusion protein) in a single well across the top of the gel. The proteins in were separated by electrophoresis and then the gel was stained with Coomassie brilliant blue. The protein band corresponding to the fusion protein was cut out of the gel, fragmented and placed in dialysis tubing containing about 2 mls of Tris-glycine buffer. The dialysis tubing was sealed and placed in a electrophoresis tank containing Tris-glycine buffer. The protein in the gel slice was electroeluted at 125 V for 5 hours. The dialysis tubing was then placed in a beaker of water (5 litres) on a magnetic stirrer in the 4°C cold room overnight to dialyse. The next day the liquid was removed from the dialysis tubing, frozen at a slant and then freeze dried. The freeze dried sample was resuspended in 300 µl of sterile water. The sample was kept frozen until needed (ie for injection into rabbits or for gel electrophoresis)

2.21 Immunising rabbits with fusion protein.

Approximately 100 µg of purified fusion protein (Protein A-Kex 2 fusion protein) was emulsified with an equal volume of Freunds complete adjuvant by vortexing (total volume of sample + adjuvant = 1 ml). The emulsified material was taken up into a 1 ml syringe and injected into rabbits. Two young New Zealand white rabbits were each immunised with this amount of material by subcutaneous injection. Five weeks after the initial immunisation a subcutaneous booster of 100 µg of fusion protein emulsified with Freunds incomplete adjuvant was given to each rabbit. The rabbits were boosted again in the same way five weeks after the first boost. Seven days after each boost a 30 ml sample of blood was taken from each animal. A sample of blood was also taken before immunisation with any fusion protein (pre-immune serum). The blood samples were all treated in the same way to obtain serum: The blood was allowed to clot by standing it at room temperature for 2 hrs in a Corex tube and then at 4°C for a further 18 hrs. The serum was poured into a clean Corex centrifuge tube leaving the majority of the clot behind and centrifuged at 5000 rpm (in a Beckman JA-20 rotor) for 10 mins. The serum was passed through a 0.22 µm Millipore filter an aliquoted into sterile Eppendorf tubes. The serum was stored frozen at -20°C except for the stock in use which was stored at 4°C to prevent continuous freezing and thawing. All experiments described were performed using the serum prepared from the second boost.

2.22 Affinity purification of antibodies using antigen immobilised on a nitrocellulose strip.

The sample containing antigen (In this case a preparation of inclusion bodies from pEX-Kex transformed pop2136 cells) was separated on a 10% preparative slab gel (the whole sample was loaded into a single well spanning the width of the gel) and transferred onto nitrocellulose (Chapter 7). The nitrocellulose was stained in 0.2% Ponceau S in 3% TCA for 5 minutes and then washed in water to remove the background staining. The band corresponding to the antigen (in this case the β-galactosidase-Kex2 fusion protein which was the largest and most abundant protein on the blot) was carefully excised from the blot using a scalpel. The Ponceau

S was removed from the affinity strip by washing in TBS several times (NB it is important to be able to determine which side of the strip has the antigen bound to it, especially once the Ponceau S is removed). The affinity strip was then incubated in blocking buffer (3% BSA in TBS) for 90 minutes in order to block any non-specific sites on the nitrocellulose and then washed in TBS. The affinity strip was placed (antigen side up) onto parafilm in a container with a lid and the immune serum (Kex2 antiserum) was placed onto the strip (as much as could be held on the strip by surface tension). The lid was placed on the box and the strip was shaken gently at room temperature for 2 hrs. The excess serum was removed from the strip and the strip was washed three times for 5 minutes each wash in TBS to remove any unbound antibodies that may have been present. The antibodies specifically bound to the antigen were removed from the strip by placing elution buffer (0.2 M glycine.HCl, pH 2.8) onto the strip (as much as could be held on the strip by surface tension) for 20 min. The elution buffer containing the eluted antibodies was removed from the affinity strip and neutralised immediately by adding an equal volume of 0.1 M Tris HCl, pH 8.5. These affinity purified antibodies were stored at 4^{o}C after the addition of 0.1% sodium azide to them. The Affinity strips were be re-used after washing in TBS, and stored at 4^{o}C in TBS, 0.1% sodium azide.

2.23 Detection of blotted proteins with antibodies (Western analysis)

Two different methods of detection were used in the experiments described in this thesis. Both methods involve the detection of a second antibody conjugated to horse radish peroxidase (HRP). In the earlier experiments the blots were developed with 4-chloro-1-naphthol and in later experiments the blots were developed with a chemi luminescent reagent (Amersham ECL system) and detected by exposing X-ray film to the blot

Detection with 4-chloro-1-naphthol

Once the proteins separated by electrophoresis had been transferred to nitrocellulose the nitrocellulose blot was placed into blocking buffer (TBS + 0.5% (v/v) Tween 20) for 90 mins. Once blocked the blot was placed into TBS + 0.05% (v/v) Tween 20 containing primary antibody (the dilution and type of primary antibody was

dependent on the particular experiment) and incubated at room temperature for 2 hrs. The blot was washed in TBS + 0.05% (v/v) Tween 20 (5 x 5 mins) and then placed into TBS + 0.05% (v/v) Tween 20 containing second antibody (a 1/2000 dilution of goat anti-rabbit HRP-conjugated IgG obtained from Sigma was routinely used). The blot was washed in TBS + 0.05% (v/v) Tween 20 and then placed into developing solution (25 ml TBS, 7 µl H_2O_2, 5 ml 4-chloro-1-naphthol (3 mg/ml) in methanol). Developing was stopped by washing the blot copiously in water and then placing in TBS.

Detection with ECL reagent

Once the proteins separated by electrophoresis had been transferred to nitrocellulose the nitrocellulose blot was placed into ECL blocking buffer (TBS + 5% (w/v) non fat dried milk (Marvel), 1% (v/v) Tween 20) for 1 hr. Once blocked the blot was placed in ECL blocking buffer containing the primary antibody (the dilution and type of primary antibody was dependent on the particular experiment). The blot was washed in TBS + 0.1% (v/v) Tween 20 (5 x 5 mins) and then placed into ECL blocking buffer containing second antibody (a 1/5000 dilution of goat anti-rabbit HRP-conjugated IgG obtained from Sigma were routinely used). The blot was then washed in TBS + 0.1% (v/v) Tween 20 (5 x 5 mins) after which it was ready to be developed. Development of the blot was performed in the dark room as it is necessary to expose the blot to X-ray film and develop the film in a short space of time. The blot was placed in 20 mls of ECL reagent (prepared as described in the Amersham booklet provided with the reagent) for 1 min. The blot was removed from the reagent and excess reagent was removed (by letting it drip off for a few seconds). The blot was wrapped in Saran wrap and placed antigen side up in an X-ray film cassette. X-ray film was exposed to the blot in the cassette for 30 seconds and then developed. The developed film was looked at and another film was exposed to the blot for a longer or shorter time (depending on how the first film looked) before being developed. Once the blot was placed in ECL reagent it was important to carry out subsequent steps quickly as the ECL reagent fades. Blots could however be re-developed using fresh ECL reagent if they are stored in TBS (ie without drying out). This method of detection was preferable to detection with chloronaphthol as it was quicker to perform, much more sensitive and a permanent record of the blot (the developed

autoradiograph) was instantly made.

2.24 Permeabilising yeast cells with Brij 58.

Yeast cells permeabilised with Brij 58 were used as the source of internal Kex2p in the *in vitro* assays for Kex2. 1 ml of yeast cells were pelleted by centrifugation in a microcentrifuge for 5 min. The cell pellet was washed in 0.9% (w/v) NaCl, harvested by centrifugation in a microfuge and the supernatant was discarded. The cell pellet was frozen at -70^{o}C for 30 min and then were allowed to thaw slowly on ice. 0.9 ml of potassium acetate pH 5.0 followed by 0.1 ml of 10% (w/v) Brij 58 were added to the thawed pellet (the cells were left on ice for 30 minutes in order for them to permeabilise). 5 µl of the permeabilised cell suspension was used in each assay for intracellular Kex2 activity.

2.25 *In vitro* assay for Kex2 activity

5 µl of the Kex2 source (either culture supernatant or cells permeabilised with Brij 58) was assayed in a 50 µl reaction mix in an Eppendorf tube as descibed by Fuller *et al* (1988):- The reaction mix contained 200 mM sodium-hepes pH 7, 1 mM $CaCl_2$, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone (TPCK), 1% (w/v) Triton X-100 and 100 µM t-butoxycarbonyl-Gln-Arg-Arg 4-methylcoumarin-7-amide (bQRR-MCA). Reaction mixtures were incubated for 30 minutes at 37^{o}C. The reactions were terminated by the addition of 0.9 ml of 0.125 M $ZnSO_4$ and 0.1 ml of a saturated $Ba(OH)_2$ solution. The precipitate formed on the addition of $Ba(OH)_2$ was removed by centrifugation for 1 minute in a microcentrifuge. The 7 amino-4-methylcoumarin (AMC) released from bQRR-MCA due to cleavage after the pair of Arginine residues (by Kex2p) was determined fluorimetrically [λ (excitation) = 385 nm, λ (emission) = 465 nm].

2.26 Microtitre assay for *K. lactis* killer toxin

The yeast strain sensitive to the toxin (LL20) was grown in YPD and diluted in toxin assay broth (18 ml YPD, 0.74 ml 0.5M citric acid, 1.26 ml 1.0 M Na_2HPO_4) to a cell density of 1 x 10^6 cells/ml. 20 µl of the supernatants of the cells to be tested for toxin production were placed into the wells of a microtitre assay plate followed by 180 µl of diluted sensitive cells. The microtitre plate was incubated at 28°C for 24 hrs. Inhibition of growth was obvious in wells containing toxin by the apparent density of cells in the wells. For a quantitative result the 200 µl of cells in the microtitre assay wells were placed into a a cuvette containing 0.8 mls of water and the A_{600} was measured. The lower the A_{600} the greater the inhibition due to the presnce of the toxin (ie there is more toxin present in the supernatant).

2.27 Assays for α factor

Both of the assays for α factor are based on the inhibition of growth of a strain of yeast that is sensitive to α factor (RC631).

Halo assay for secreted α factor

The growing yeast strain RC631 was inoculated to a density of 10^6 cells/ml into YPD + 0.8% (w/v) Bacto agar (no warmer than 50°C) and poured immediately ont YPD plates to create a lawn of sensitive cells. The plates were dried in a 30°C incubator and then the strains to be tested for α factor secretion were patched onto the seeded lawn. The plates were incubated at 30°C for approximately 36 hrs, until the sensitive strain of yeast had grown and the halos of inhibition of growth could clearly be seen. The size of the halo is an indication of the amount of α factor being produced by the seeded strain, with a large halo indicative of a large amount of α factor

Microtire assay for secreted α factor

A growing culture of the sensitive yeast strain RC631 was diluted to a density of approximately 10^5 cells/ml. 100 µl of the supernatant (or a dilution of the supernatant) to be tested for the presence of α factor was placed into the well of a microtitre assay plate. This was followed by 100 µl of the diluted sensitive cell culture. The microtitre assay plate was incubated overnight (18 hrs) at 30°C. The density of the sensitive cells was measured by placing the 200 µl of cells in the assay well into a cuvette containing 800 µl of water and then measuring the A_{600}. The lower the A_{600} the greater the amount of α factor in the supernatant. Thus the density to which the sensitive cells grew to could be used to estimate the relative amounts of α factor in the media of different strains.

2.28 Processing yeast cells for immunocytochemistry using LR white resin.

The processing of all of the strains of yeast used for immunocytochemistry was by a method obtained from Dr R. Wright, Biochemistry department, Barker Hall, University of California, Berkeley (personal communication):-

Fixation of the yeast cells.

Gross morphological alterations and changes in the presence and/or location of proteins has been reported when yeast are subjected to centrifugal forces. To avoid any such affects in this method, the fixative is added directly to the growing culture of yeast.

(1) A 1/10 volume of 10x prefixative solution (10% gluteraldehyde, 2% methanol free formaldehyde, 0.4M potassium phosphate pH7) was placed into a centrifuge tube.

(2) The early log phase yeast culture (50ml) grown in selective medium was poured

rapidly into the fixative and allowed to sit at room temperature for 5 minutes.

(3) The cells were pelleted and resuspended in 1/10 volume of ice cold 1x prefixative (1% gluteraldehyde, 1% methanol free formaldehyde in 0.04M potassium phosphate pH7) and allowed to complete fixation for 30 minutes.

(4) Excess fixative was removed by three buffer washes (ie 0.04M potassium phosphate pH7) leaving the cell suspension in each change of buffer for at least 5 minutes.

Periodate treatment

(1) The washed cell pellet was washed in 5 ml of freshly prepared 1% sodium metaperiodate (aqueous) and allowed to incubate at room temperature for 15 minutes. The samples became clumpy at this point which is an advantage in later stages. The cells were pelleted and washed once in phosphate buffer.

(2) The pellet was resuspended in 50mM ammonium phosphate to block free aldehyde sites. After 15 minutes the cells were washed twice with distilled water.

Dehydration

(1) The cells were resuspended in 10ml of 50% ethanol and then "spun" in a conical glass centrifuge tube for 5 minutes to pellet the cells (the use of glass is important for maximising clumping of the pellet into aggregates of convenient size).

(2) The supernatant was replaced with 1ml of 70% ethanol. At this point the cells formed firm pellets that could be gently dislodged with a pasteur pipette into small fragments. Aliquots containing 10-20 small "chunks" were dispensed into eppendorf tubes containing 70% ethanol. These aliquots can be stored at -20^{o}C for several months but in this case subsequent processing was carried out immediately.

(3) The tubes containing the samples were placed on a rotating drum for 5 minutes. Dehydration was completed by subsequent incubations (5 minute rotations) in 95% and 3 changes in 100% ethanol. For the 100% ethanol incubations a new bottle of

ethanol was opened to ensure the absence of water.

Infiltration

(1) The last 100% ethanol incubation was replaced with a solution of 2 parts ethanol to 1 part resin (LR white). The eppendorfs were returned to the rotator for 1 hr.

(2) The resin mix was replaced with a 1:1 resin:ethanol mix and the samples were rotated for 1 hr.

(3) The resin mix was replaced with a fresh 1:1 mix and the samples were rotated uncapped overnight in a fume hood. The ethanol slowly evaporates thus increasing the resin concentration gradually.

(4) The next day the residual resin was replaced with fresh 100% resin and the samples rotated for an hour. The resin was changed and the samples placed under vacuum (20 psi) for 15 minutes to degas, then they were placed back on the rotator for 1 hr.

Embedding

(1) Small pellet fragments were removed from the eppendorf tubes using a tooth pick and placed into gelatin capsules filled with resin.

(2) The sample was allowed to sink to the bottom of the capsule, positioned in the centre and then placed under vacuum for 15 minutes.

(3) Polymerisation was accomplished by leaving the gelatin capsules in an incubator at 45^{o}C-50^{o}C for 2 days.

Grid preparation and sectioning

Sections from the LR white resin blocks were cut by the Department of Pathology, University of Edinburgh and placed onto gold EM grids. The sections were air dried on

the grids for two days.

Immunolabelling

For this technique it is essential to use affinity purified antibodies derived from polyclonal antiserum as when crude antiserum is used contaminating antibodies can produce severe problems in interpretation. Background labelling of the cell wall, vacuole and nucleus are especially problematical. As the affinity purified Kex2 antibodies appear to be specific for Kex2p in Western analysis it was decided that these antibodies would be good enough to use as the primary antibody in immunolabelling experiments of the different strains of yeast expressing the various forms of the truncated Kex2p and of control strains.

Setting up the incubation chamber

(1) The incubation chamber consisted of a box with a tight fitting lid, of sufficient size to contain all of the grids and solution droplets. Moist paper towels were placed in the incubation chamber in order to maintain humidity throughout the labelling steps.

(2) Parafilm was positioned in the box and small coloured adhesive dots (sticky dots) labelled with the identity of the grid were placed down the left side of the parafilm. Across the top of the parafilm 4 sticky dots were also positioned. The first and third represented positions at which droplets of blocker would be placed. The second represented the position of the droplet of primary antibody (affinity purified Kex2 antibody) and the fourth the position of the droplet of secondary antibody (goat anti rabbit gold conjugated antiserum:-10nm gold particles).

Reagents

PBST (140mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4 and 0.5% Tween 20) was used throughout the immunolabelling protocol for all washes and as the vehicle for the blocker. The blocking solution was PBST containing 2% bovine albumin. All solutions were filtered through a 0.22 μm filter (Acrodisc) before use.

Both primary and secondary antibodies were diluted in blocking solution. The best dilution factor for the batch of affinity purified Kex2 antibodies was found to be 1/50 by using a dilution series. The gold conjugated second antibody was adjusted to A_{525} = 0.13.

Experimental procedure for immunolabelling

(1) 20µl droplets of blocking solution were positioned at the appropriate positions on the parafilm sheet. The appropriate grid was submerged in the blocking solution and allowed to incubate for 15 minutes at room temperature.

(2) After blocking the grid was removed from the blocking solution, touched onto a Kimwipe to remove excess fluid and then submerged in a 20µl droplet of primary antibody for 2 hrs at room temperature.

(3) The grids were next washed 3 times for 5 minutes in PBST (0.5ml). The washes were performed in a porcelein dish shaking at a speed so that the solution was moving as rapidly as possible without spilling.

(4) The grids were transferred from the washes to a second drop of blocking solution for 15 minutes and the to a 20µl drop of secondary antibody for 1 hr at room temperature.

(5) Washes were performed as in (3) and after the final PBST wash the grids were dipped 10 times into distilled water in a 5ml beaker with a rapid up and down motion. This step was to remove salts that may crystallise on the grid and obscure the view under the electron microscope. The grids were allowed to air dry for 2 days before staining

(6) The grids were stained with 2% aqueous uranyl acetate for 1-5 minutes and in Reynolds lead citrate for 30 seconds by the Department of Pathology, University of Edinburgh.

Chapter three.

Biogenesis of the *K. lactis* killer toxin.

3.0 Introduction

The study of intermediates in the maturation of proteins in yeast has been aided greatly by the use of conditional *sec* mutants, isolated by Novick *et al* (1980). These *sec* mutants are blocked in secretion and, at the restrictive temperature of 37°C, accumulate intermediate forms of proteins in specific organelles of the secretory pathway (depending on the particular mutant). The processing events that these intermediates have undergone are an indication as to those processing enzymes to which they have been exposed.

The *Kluyveromyces lactis* killer toxin is secreted by strains of *Saccharomyces cerevisiae* harbouring the linear DNA plasmids k1 and k2. It should therefore be possible to use the well-established genetics of *S.cerevisiae,* and the *sec* mutants in particular, to monitor the processing and assembly of the *K.lactis* killer toxin as has been done for α factor (Julius *et al* 1984), invertase (Esmon *et al* 1981) etc. One of the processing events that occurs in the maturation of the *K.lactis* killer toxin is the proteolytic cleavage of the 130K αβ precursor polypeptide to give rise to the mature 99K α and 30K β polypeptides. The Kex2p of *S.cerevisiae* has been implicated in the this processing event as the cleavage of the αβ precursor is known to be at the carboxyl side of Lys-Arg sequence which is known to be cleaved by Kex2p in other proteins (ie prepro α factor). Furthermore *K.lactis* possesses a functional homologue (the *K.lactis* Kex1 protease) of the Kex2 protease of *S.cerevisiae* that was isolated as being deficient in processing the *K.lactis* killer toxin, and which also has sequence similarity to Kex2p (Tanguy-Rogeau *et al* 1988). In this chapter I will describe experiments which verify the involvement of Kex2p in the processing of the αβ precursor of the toxin in *S.cerevisiae*. Also the timing of processing and assembly of the toxin in the secretory pathway will be discussed.

3.1 Involvement of Kex2p in the processing of *K.lactis* killer toxin

S.cerevisiae strain F102-2 harbours the linear DNA plasmids k1 and k2 (for full genotype see Table. 2.1) and secretes a functional killer toxin that inhibits the growth of a sensitive strain of *S.cerevisiae* called LL20. To show the involvement of Kex2p in the processing of the *K.lactis* killer toxin it was decided to replace the *KEX2*

gene of F102-2 with a non-functional *KEX2* gene which is disrupted with a selectable marker, the *LEU2* gene (*kex2::LEU2*). The *kex2::LEU2* disruption was obtained as a Bam HI/Sph I fragment in a plasmid called pGA1070 (supplied by Dr. G. Ammerer). The first step in this gene replacement was to digest the plasmid pGA1070 with Bam HI and Sph I and purify the Bam HI/Sph I fragment (fig. 3.1). This purified fragment was transformed into F102-2 with selection for Leu^+ colonies (untransformed F102-2 is Leu^-). These Leu^+ transformants should have had their chromosomal copy of the *KEX2* gene replaced with the *kex2::LEU2* disruption from pGA1070 (fig. 3.1). The transformants were checked for the replacement of the *KEX2* gene with the *kex2::LEU2* disruption by Southern analysis of the chromosomal DNA of the transformants (fig. 3.2).

The Southern analysis (fig. 3.2) showed that three of the transformants (2, 5 and 6) all appeared to have had the chromosomal copy of the *KEX2* gene replaced with the *kex2::LEU2* disruption from pGA1070. Three of the transformants (1, 3 and 4) appeared to possess both the normal chromosomal copy of the *KEX2* gene and the *kex2::LEU2* disruption. This was possibly because the *kex2::LEU2* disruption had integrated somewhere in the chromosome but not at the *KEX2* locus. Another possibility was that when the transformants 1, 3 and 4 were being picked a mixture of colonies with and without the gene replacement were picked instead of a single colony. A third possibility is that the Bam HI/Sph I fragment has somehow become recircularised in the yeast and has a fortuitous origin of replication so that it can be maintained in the cells under selection. Transformants 2, 5 and 6 were assumed to be $Kex2^-$ as they unambiguously have the chromosomal copy of *KEX2* replaced with the *kex2::LEU2* disruption.

Strain F102-2, together with the six transformants previously checked for replacement of the normal chromosomal copy of the *KEX2* gene with the *kex2::LEU2* disruption, were tested to see whether they secreted active *K.lactis* killer toxin or not:- 5ml cultures of F102-2 and the six transformants were grown overnight (16 hr) in YPD medium at 30°C. All of the cultures of yeast had reached stationary phase by the next morning. 1ml of cells from each of the cultures was harvested by centrifugation and the cells were kept. The supernatants from each of the spun cultures was also kept and were assayed by microtitre assay (see Materials and Methods) for *K.lactis* killer toxin activity. The harvested cells were checked for the

Fig 3.1 Replacement of the chromosomal copy of *KEX2* in F102-2 with a non functional disrupted *KEX2* gene (*kex2::LEU2*).

The Bam HI/Sph I fragment of pGA1070 (supplied by Gustav Ammerer) has the majority of the *KEX2* gene replaced with the *LEU2* gene (*kex2::LEU2*) but the flanking regions are intact. Yeast strain F102-2 was transformed with the *kex2::LEU2* disruption from pGA1070 with selection for Leu$^+$ transformants. Homologous recombination of the regions flanking the *kex2::LEU2* disruption in the Bam HI/Sph I fragment and the regions flanking the chromosomal copy of the *KEX2* gene results in the replacement of the chromosomal copy of the *KEX2* gene with the *kex2::LEU2* disruption. The resulting strain with the recombinant chromosome was named PWYS1 and does not contain a functional *KEX2* gene. The restriction sites shown are:- Bam HI (B), Bgl II (Bg), Eco RI (E), Hind III (H), Sph I (S) and Pvu II (P).

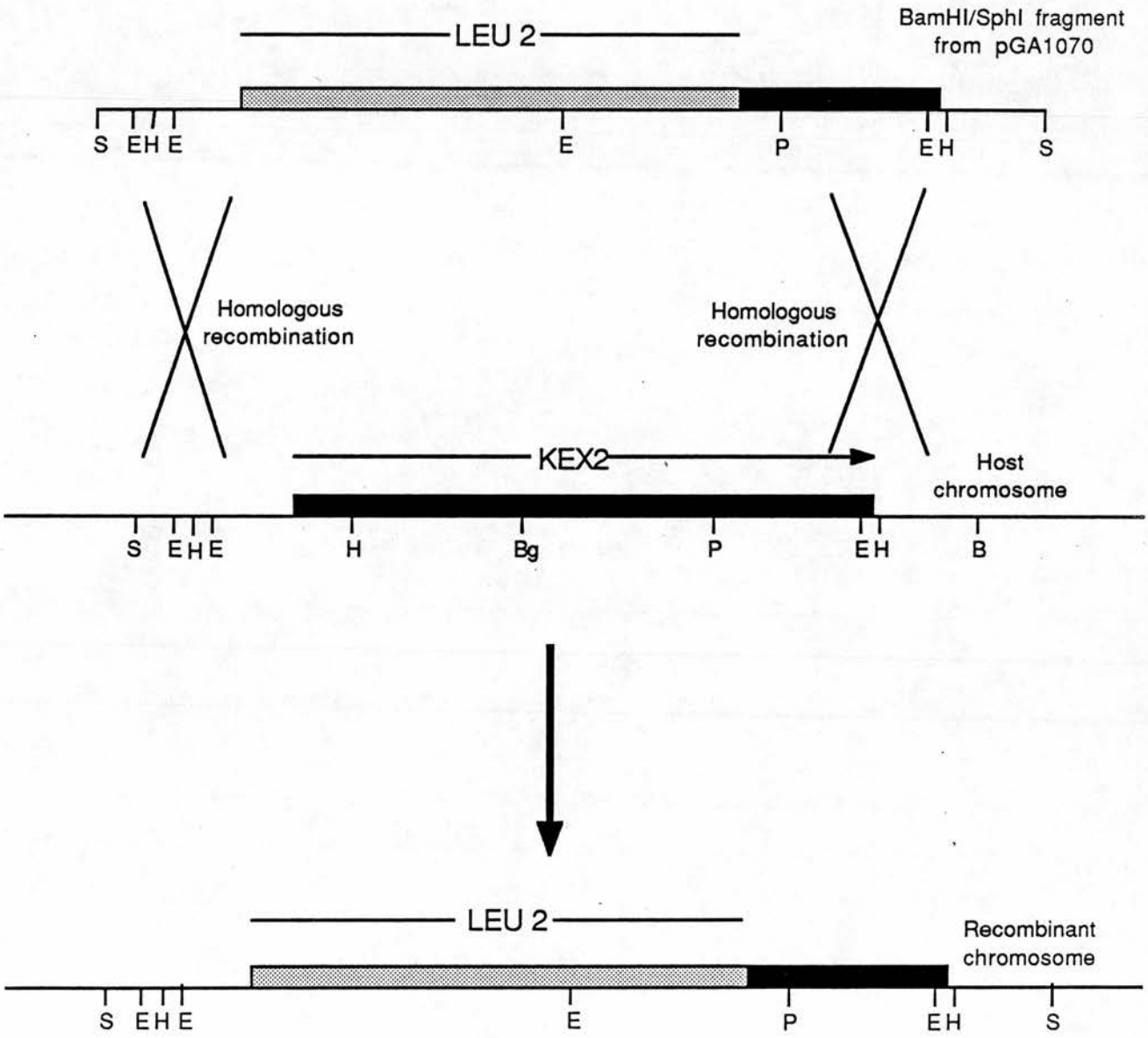

LEU 2
BamHI/SphI fragment
from pGA1070
S EH E
E
P
EH
S
Homologous
recombination
Homologous
recombination
KEX2
Host
chromosome
S EH E
H
Bg
P
EH
B
LEU 2
Recombinant
chromosome
S EH E
E
P
EH
S

Fig 3.2 Southern analysis of F102-2 and F102 transformants.

Total DNA from F102-2 and F102-2 transformed with the *kex2::LEU2* disruption fragment was digested with Eco RI and electrophoresed on a 0.8% agarose gel. The DNA was blotted onto nitrocellulose. Southern analysis was performed using a radiolabeled DNA probe. The DNA fragment used as the probe is shown in the figure and consists of a 3.6 kb Eco RI fragment of the *KEX2* gene (purified from plasmid pGA714, supplied by Dr. G Ammerer). The lanes of the autoradiograph are as follows:-

Lane 1. F102-2

Lane 2. F102-2 transformant n^{o} 1

Lane 3. F102-2 transformant n^{o} 2

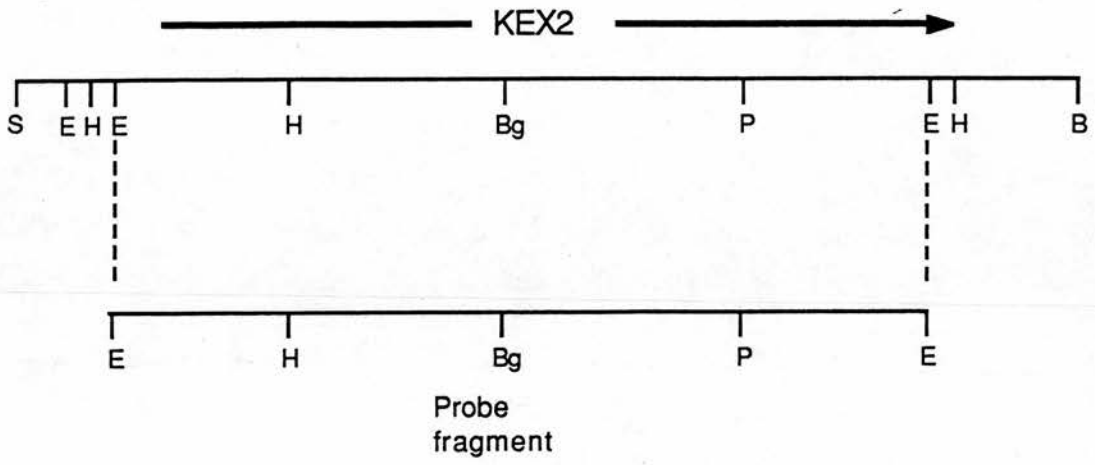
Lane 4. F102-2 transformant n^{o} 3

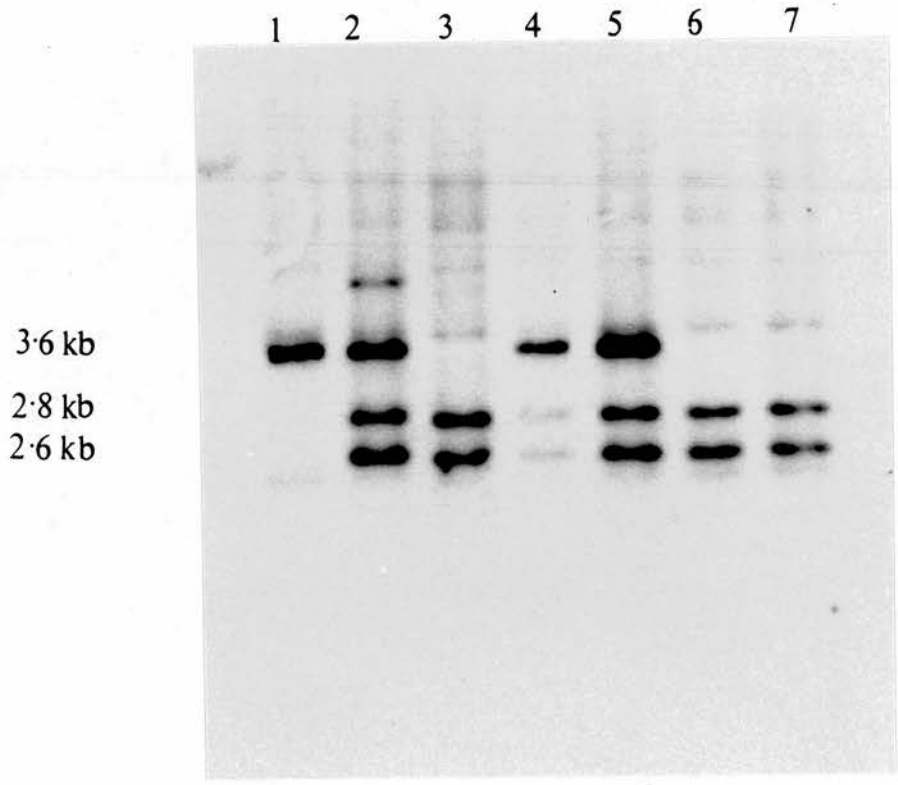
Lane 5. F102-2 transformant n^{o} 4

Lane 6. F102-2 transformant n^{o} 5

Lane 7. F102-2 transformant n^{o} 6

The 3.6 kb band on the autoradiograph is indicative of the *KEX2* gene. The 2.8 kb and 2.6 kb bands are indicative of the *kex2::LEU2* disruption fragment being present (when *kex2::LEU2* is digested with Eco RI, two fragments of 2.8 kb and 2.6 kb are released; Both of them are recognised by the *KEX2* probe).


KEX2
S
E H E
H
Bg
P
E H
B
E
H
Bg
P
E
Probe
fragment


1
2
3
4
5
6
7
3·6 kb
2·8 kb
2·6 kb

presence of linear plasmids by *in situ* lysis electrophoretic analysis (see Materials and Methods).

The results of the killer toxin assay (Table 3.1) show that F102-2 and transformants 1, 3 and 5 all secreted active killer toxin as they inhibited the growth of LL20 the sensitive strain. Transformants 2, 5 and 6 did not appear to secrete active killer toxin as they did not inhibit the growth of LL20. These results suggest that when the normal *KEX2* gene is present (ie in F102-2 and transformants 1, 3 and 4) the *K.lactis* killer toxin is processed and secreted into the culture medium in an active form. However when the normal chromosomal copy of the *KEX2* gene is replaced with the *kex2::LEU2* disruption (ie in transformants 2, 5 and 6) to make the strain Kex2$^-$, there is no detectable *K.lactis* killer toxin activity in the supernatants. These results suggest that an active Kex2p is required for the processing and/or secretion of *K.lactis* killer toxin from *S.cerevisiae*.

The *in situ* lysis gel (fig. 3.3) shows that F102-2 and all of the transformants contained the linear plasmids and therefore were capable of producing *K.lactis* killer toxin. The amount of DNA in each of the lanes varies even though approximately the same amount of cells from each of the cultures were treated with zymolyase and loaded onto the gel. It appears that lanes corresponding to F102-2 and transformants 1, 3 and 4 contained more linear plasmid DNA than transformants 2, 5 and 6. Thus for some reason the Kex2$^-$ strains appear to have less linear plasmid DNA on the gel than Kex2$^+$ strains. One explanation could be that Kex2$^-$ mutants do not form spheroplasts as readily as Kex2$^+$ strains and therefore when placed into the wells of the in situ lysis gel only a proportion of them are lysed and released their DNA content into the gel. An interesting observation is that Kex2$^-$ mutants tend to clump together when grown in liquid medium to a greater extent than Kex2$^+$ strains. This clumping could possibly effect the efficiency of spheroplast formation as all the cells in the clump may not be accessible to digestion by zymolyase. Another possible explanation for the differing DNA contents on the gel could be that Kex2$^-$ mutants are more susceptible to lysis during spheroplast formation and a high proportion lyse to release their DNA content before being loaded into the wells of the *in situ* lysis gel.

TABLE 3.1 Results of the microtitre assays for secreted killer toxin in cell supernatants

Supernatant from strain	% of control growth
F102-2	22%
F102-2 transformant 1	24%
F102-2 transformant 2	100%
F102-2 transformant 3	29%
F102-2 transformant 4	28%
F102-2 transformant 5	103%
F102-2 transformant 6	102%

The results of the toxin assays of the cell supernatants from the different strains are expressed as a percentage of the density to which LL20 (the toxin sensitive strain) grows when no toxin is present (ie cell supernatant is replaced with YPD in the microtitre assay). Hence a low percentage indicates that active toxin was present in the cell supernatant and a high percentage indicates that that very little (or no) active toxin is present in the cell supernatant.

Fig 3.3 *In situ* lysis analysis of F102-2 and F102-2 transformants

The strain F102-2 and F102-2 transformed with the kex2::LEU2 disruption fragment were analysed by *in situ* lysis gel electrophoresis for the presence of the linear plasmids k1 and k2.

Lane 1. F102-2 transformant n^{o} 1

Lane 2. F102-2 transformant n^{o} 2

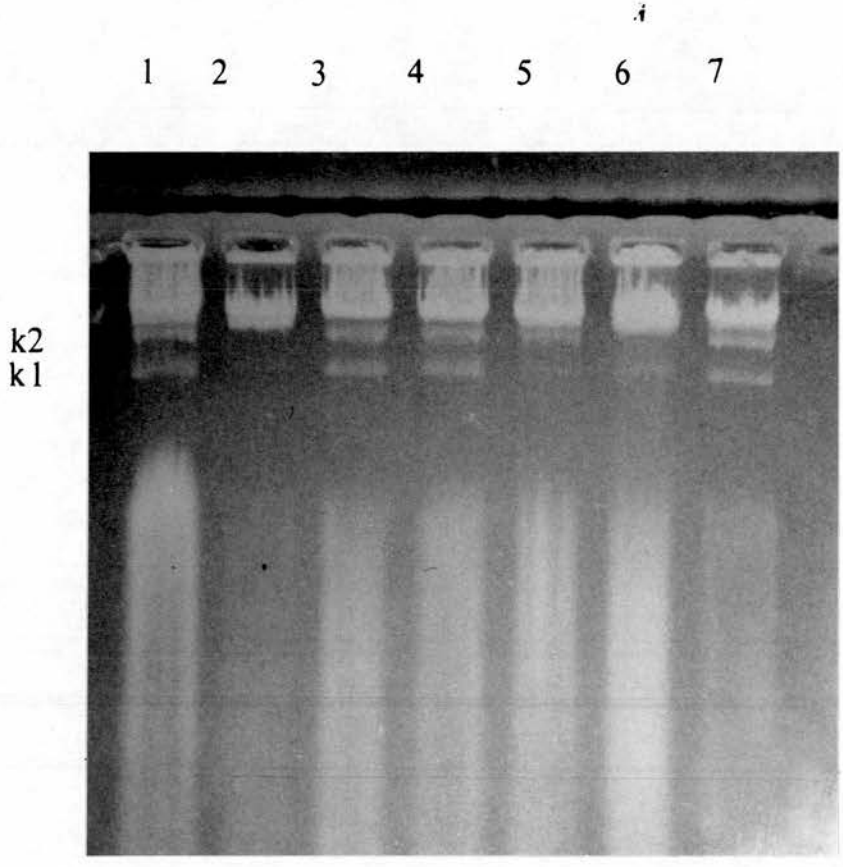
Lane 3. F102-2 transformant n^{o} 3

Lane 4. F102-2 transformant n^{o} 4

Lane 5. F102-2 transformant n^{o} 5

Lane 6 . F102-2 transformant n^{o} 6

Lane 7. F102-2


1 2 3 4 5 6 7
k2
k1

3.2 Processing and assembly of the K.lactis killer toxin in the secretory pathway

The linear DNA plasmids k1 and k2 were placed in *S. cerevisiae* strains with different genetic backgrounds by a method called cytoduction (Zakharov et al 1969). Strains MRY101, MRY102 and MRY103 (M. Romanos and A. Boyd unpublished) contained the linear plasmids in *sec18*, *sec14* and *sec1* backgrounds respectively. These strains are all temperature sensitive for growth and should accumulate specific secretory organelles at the restrictive temperature of 37°C because of their *sec* genotypes. MRY101 should accumulate ER, MRY102 should accumulate Golgi and MRY103 should accumulate secretory vesicles. It was intended to examine the accumulation of intermediates in the processing and assembly of the *K. lactis* killer toxin in these strains and by analysing the intermediates to deduce the order of processing and assembly of the toxin.

3.3 Analysis of the toxin accumulated in MRY101, MRY102 and MRY103 at the restrictive temperature.

MRY101 is a *sec18* mutant that contains the linear DNA plasmids k1 and k2. At the permissive temperature of 26°C MRY101 secretes active killer toxin. However at the restrictive temperature of 37°C MRY101 is arrested in growth and according to its genotype should accumulate ER and ER forms of secretory proteins. Therefore ER modified forms of the *K.lactis* killer toxin should be accumulated in MRY101 at 37°C. Analysis of the toxin accumulated in MRY101 at 37°C should indicate the processing and assembly events that have taken place before the *sec18* block.

Strain MRY101 was grown in 10 ml of labeling medium to a cell density of 2×10^7 cells/ml at 26°C. The culture was then shifted to 37°C in order to impose the *sec18* block. After 10 minutes at 37°C radioactive label ([^{35}S]-methionine) was added to the culture and labelling allowed to proceed for 1 hr. After 1 hr the labelling was stopped by addition of sodium azide and extracts were made from the labelled cells. Immunoprecipitations using polyclonal antibodies to different subunits of the toxin were carried out on the labeled cell extracts (For details of labelling, cell extract and immunprecipitation protocols see Materials and Methods). An antibody specific to the α subunit of the toxin (anti α antibody) and an antibody specific to the γ

subunit of the toxin (anti γ antibody) were used to immunoprecipitate material from MRY101 cell extracts labeled at 37°C. The material specifically immunoprecipitated was run on a reducing 10% SDS PAGE, and processed for fluorography (see Materials and Methods)

The fluorograph (fig. 3.4) shows that the α-specific antibody specifically precipitates a protein of approximate molecular weight 130K. This protein is presumed to be the αβ precursor of the toxin as it is the predicted size of the αβ precursor and the anti-α antibody should recognise the αβ precursor as well as the mature α subunit. The γ specific antibody specifically precipitates a protein with an approximate molecular weight of 30K. This protein is presumed to be the γ subunit of the toxin as it is in the correct size range to be the γ subunit and is the only subunit of the toxin that should be specifically precipitated by the anti-γ antibody. Pre-immune sera from both rabbits used in the preparation of the anti-α antibodies and the anti-γ antibodies do not specifically precipitate any polypeptides (fig. 3.4). There does not appear to be any co-precipitation of the γ subunit with the αβ precursor when anti-α antibodies are used in the immunoprecipitation and conversely there does not appear to be any co-precipitation of the αβ precursor with the γ subunit when anti-γ antibodies are used in the immunoprecipitation. A number of different methods for making labeled cell extracts in combination with different methods for immunoprecipitation have been tried and co-precipitation of the αβ precursor and the γ subunit has not been detected with any of them (data not shown). The simplest explanation for this observation is that the γ subunit is not covalently attached to the rest of the toxin by the disulphide bond known to exist in the fully assembled toxin until after the position in the secretory pathway at which *sec18* blocks secretion. This explanation would, however, go against the accepted view that disulphide bonds form in the ER (Freedman 1984). Another explanation could be that in making the cell extract or in the immunoprecipitation procedure the disulphide bonds are reduced and the toxin falls apart. A third explanation, less likely given that the antiserum is polyclonal is that the antibodies used only recognise free subunits and not assembled toxin complexes. The fact that a 130K protein (the αβ precursor) is the only protein specifically precipitated using anti α antibodies indicates that the

Fig. 3.4 Immunoprecipitations of toxin subunits from a *sec18* mutant labeled at the restrictive temperature.

Strain MRY101 was labelled at 37°C, extracts made and immunoprecipitations performed as described in Materials and Methods. Immunoprecipitates were precipitated either with *Staphylococcus aureus* (*Staph. A*) cells or protein A-Sepharose (PAS). The antisera used in the immunoprecipitations were anti α immune serum anti γ immune serum (both raised against β-galactosidase fusion proteins by M. Stark and A. Boyd unpublished). The pre immune sera (α-PI and γ-PI) came from the same rabbits in which the immune sera were raised, before immunisation.

Lane 1. ^{14}C-labelled marker proteins.

Lane 2. MRY101 extract immunoprecipitated with anti-α immune serum (*Staph A*)

Lane 3. MRY101 extract immunoprecipitated with α-PI serum (*Staph A*)

Lane 4. MRY101 extract immunoprecipitated with anti-γ immune serum (*Staph A*)

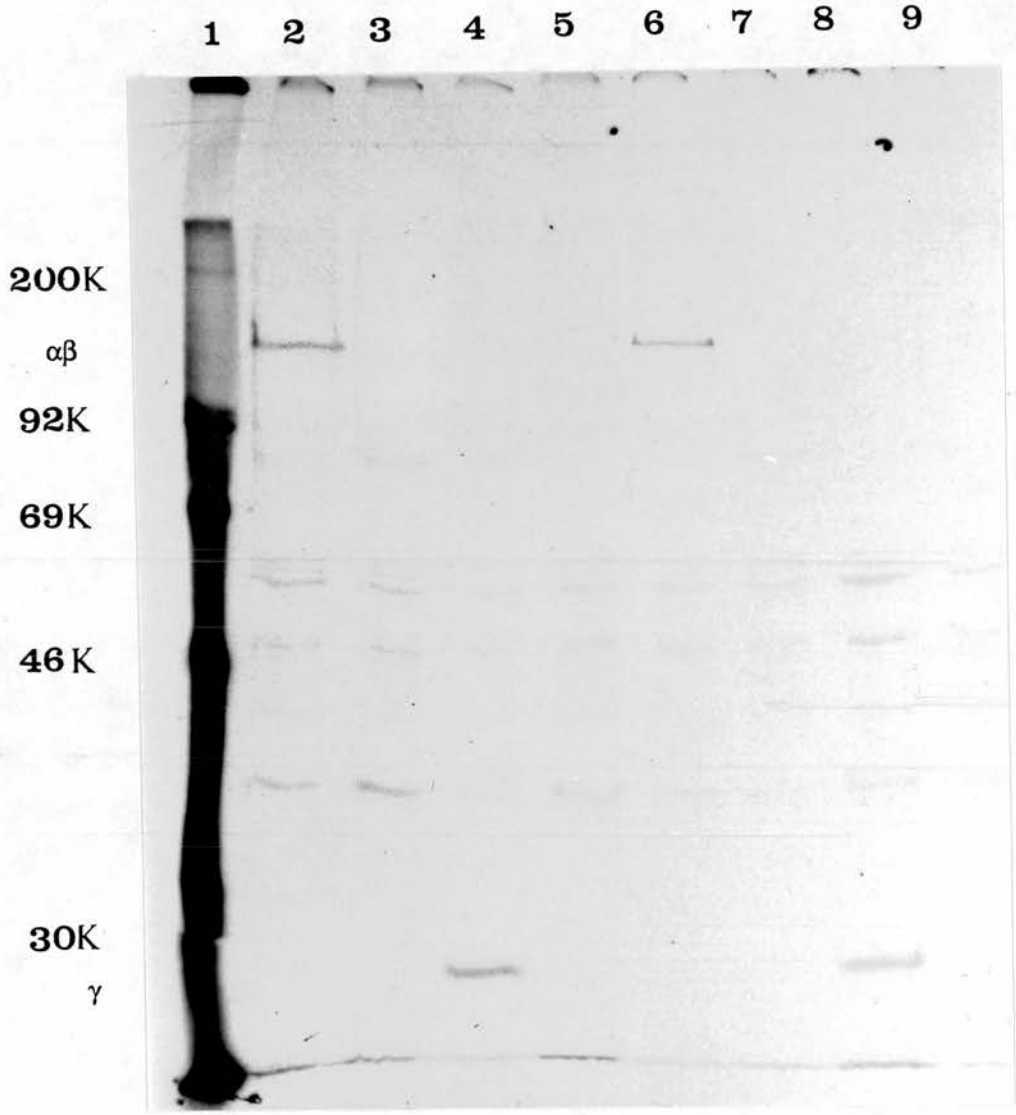
Lane 5. MRY101 extract immunoprecipitated with γ-PI serum (*Staph A*)

Lane 6. MRY101 extract immunoprecipitated with anti-α immune serum (PAS)

Lane 7. MRY101 extract immunoprecipitated with α-PI serum (PAS)

Lane 8. MRY101 extract immunoprecipitated with anti-γ immune serum (PAS)

Lane 9. MRY101 extract immunoprecipitated with γ-PI serum (PAS)


1
2
3
4
5
6
7
8
9
200K
αβ
92K
69K
46K
30K
γ

toxin being accumulated in MRY101 labeled at 37°C has not been proteolytically processed by Kex2p to release the α and β subunits. This suggests that cleavage by Kex2p does not normally occur until a position after that at which the *sec18* block has its effect, as expected. The αβ precursor can also be specifically precipitated in immunoprecipitations from MRY101 cell extracts labelled at 26°C using anti α antibodies but the amount is much less than when labelling is at 37°C (data not shown). The αβ precursor precipitated from MRY101 cells labelled at 26°C could be en route through the secretory pathway or the *sec18* defect may affect exit from the ER even at 26°C but to a lesser extent than the total block at 37°C. There is evidence that *sec18* mutants are defective, even at the permissive temperature by the synthetic lethality of double mutants (Kaiser and Schekman 1990).

In situ lysis analysis of MRY103 revealed that it did not contain the linear plasmids k1 and k2 as it was supposed to (data not shown). The strain still retained its temperature sensitive phenotype which suggested that it was a *sec1* mutant as expected but the linear plasmids were not present. The reason for this is not known but a possibility could be that the linear plasmids are not stably maintained in a *sec1* background and the plasmids are lost at a high frequency from the strain. Although the original MRY103 (made by Dr M Romanos) contained the linear plasmids, during routine re-streaking of the strain, colonies may have been selected that fortuitously lacked the linear plasmids because of plasmid loss due to instability in a *sec1* background.

MRY102 is a *sec14* mutant that contains the linear plasmids k1 and k2. At the permissive temperature of 26°C this strain secretes active killer toxin. However at the restrictive temperature of 37°C MRY102 is arrested in growth and according to its genotype should accumulate Golgi and Golgi forms of secretory proteins. Therefore Golgi-modified forms of the *K.lactis* killer toxin should be accumulated in this strain at 37°C. Analysis of the toxin accumulated in MRY102 at 37°C should indicate the processing and assembly events that have taken place before the sec14 block. Labeling and immunoprecipitation experiments have been performed on MRY102 using exactly the same methods as with MRY101. No specific proteins were precipitated with either the anti α or anti γ antibodies (data not shown). The reason for this is not known as the linear plasmids were present and the strain was

temperature sensitive for growth.

3.4 Concluding remarks

In this chapter I have confirmed the involvement of Kex2p in the processing of the *K.lactis* killer toxin by replacing the *KEX2* gene with the *kex2::LEU2* disruption and showing that linear plasmid bearing strains with this disruption do not secrete active killer toxin. Also I have demonstrated that cleavage of the αβ precursor by Kex2p has not occured before the stage in the secretory pathway at which *sec18* exerts its effect (ie before transport to the Golgi). This was expected as Kex2p is reported to be a resident of the Golgi apparatus. The γ subunit of the killer toxin does not appear to have become covalently attached to the αβ precursor before the position in the secretory pathway at which *sec18* exerts its effect (a question that has not previously been examined), although there is some doubt as to whether this result is real or an artifact due to the experimental techniques used. Analysis of intermediates of processing and assembly of the killer toxin accumulated in Golgi and secretory vesicles proved unsuccessful due to problems with the plasmid-bearing strains.

Chapter four.

Construction of plasmids for the expression in yeast of truncated forms of the Kex2 endopeptidase containing ER retention signals.

4.0 Introduction

As secretory proteins proceed through the secretory pathway they are processed in an ordered fashion by a variety of specific enzymes in distinct compartments of the pathway. What is the reason for this sequential modification of secretory proteins in separate compartments and is the order in which the processing events occur important for the correct biogenesis and secretion of secretory proteins? Because of the complexities of the secretory pathway and the processes that take place in it there may not be a simple explanation for the compartmentalised processing of secretory proteins but the following or a combination of the following may provide some of the answers:- The order of processing events may be important in helping a protein attain its final folded configuration. On the other hand the ordered processing may be a consequence of the processing enzymes needing to be in separate compartments with different environments in order to function efficiently. It is also possible that some processing events require another processing event to have taken place previously in order for them to occur. For example modifications to N-linked oligosaccharides on polypeptides are dependent on the core oligosaccharide having being transferred to the polypeptide in the first place. It is not so clear cut whether other processes are dependent on other events having occurred or not. For example it is quite possible that proteolytic processing events are completely independent of processes such as glycosylation. Alternatively glycosylation could mask potential processing sites, or it is possible that glycosylation causes a protein to fold in such a way that sites not normally exposed are exposed to proteolytic processing enzymes, thus in these cases proteolytic processing is not independent of glycosylation.

Much experimental work has been carried out on the order of events that take place in the secretory pathway during the biogenesis of secretory proteins. Our aim was to alter the order of events in the secretory pathway of yeast and see what effect that this had on the biogenesis and secretion of secretory proteins. The particular event that we were interested in altering was the proteolytic processing of secretory proteins by Kex2p. We aimed to do this by relocating the Golgi enzyme Kex2p to the ER, hoping to get premature cleavage of secretory proteins in the ER by this relocated Kex2p. In this chapter I will describe the strategy chosen to localise Kex2p to the ER and the construction of plasmids that will hopefully enable the relocation of Kex2p in yeast.

4.1 The strategy used to localise Kex2p to the ER

The Kex2 endoprotease of *S.cerevisiae* is membrane-bound and resident in the Golgi apparatus. It has been shown that the removal of 200 amino acids from the C-terminus of the Kex2p which removes the transmembrane domain does not effect the specificity of the enzyme in an *in vitro* assay (Fuller *et al* 1988). Our strategy was to attach specific amino acid sequences that have been used to localise proteins to the ER (Pelham *et al* 1988) to the C-terminus of a truncated Kex2p with the transmembrane domain removed. These truncated polypeptides with the different C-termini would then be expressed in yeast to look at their effects on the biogenesis of secretory proteins. During the course of this project we were encouraged by an observation that expression of a soluble CD4 receptor (normally a membrane bound protein) mutated to contain a specific retention signal for the ER, blocks the secretion and surface expression of the envelope glycoprotein (gp20/41) of human immunodeficiency virus (HIV-1) (Buonocore and Rose 1990). These observations may have implications in Aids research (discussed by Buonocore and Rose 1990) but our interest was that a soluble version of a protein that was normally membrane bound had apparently been specifically retained in the ER by the attachment of retention signals.

4.2 Construction of vectors enabling the expression of a truncated Kex2p with different C-termini in yeast

The full length *KEX2* gene including promoter regions was obtained as a Bam HI/Sph I fragment (fig. 4.1) cloned into YEp13 (This plasmid, pGA714 was supplied by Dr. G Ammerer). The first step in the construction was to clone an Eco RI/Pvu II fragment from pGA714 (fig. 4.1) into pK19. The Eco RI/Pvu II fragment (*kex2'*) codes for a truncated Kex2 polypeptide (Kex2'p) (fig. 4.1) and was cloned into pK19 (fig. 4.2). The resulting plasmid was called pKkex' (fig. 4.2).

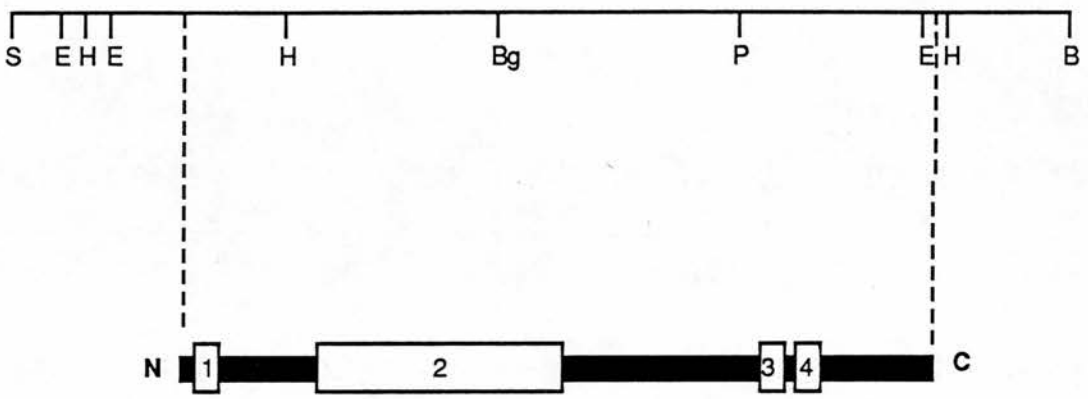
Oligonucleotides were designed (fig. 4.3) that would place the amino acid sequences HDEL and KDEL in frame at the extreme C-terminus of Kex2'p. HDEL has been shown to localise proteins to the ER in yeast when placed at the extreme C-terminus and KDEL does the same in animal cells. The KDEL signal is not thought to be an efficient signal for localising proteins to the ER in yeast. The oligonucleotides were designed

Fig. 4.1 The *KEX2* gene the *kex2'* gene and the polypeptides that they encode

(A) The diagram shows the SphI (S)/BamHI (B) fragment from pGA714, the other sites shown are Eco RI (E), Hind III (H), Pvu II (P) and Bgl II (Bg). The fragment is approximately 5 kb and encodes a polypeptide (Kex2p) 814 amino acids in length. Features of the deduced amino acid sequence are:- (1) A signal sequence. (2) A domain of homology to subtilisin that is thought to be the catalytic site. (3) A Ser/Thr rich domain thought to be an area of extensive O-linked glycosylation. (4) A transmembrane domain.

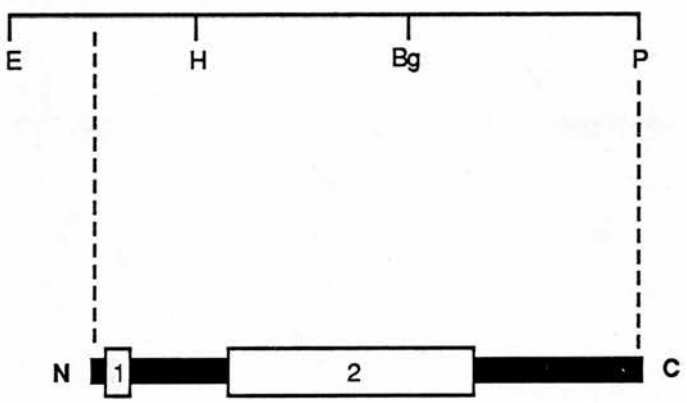
(B) The diagram shows the Eco RI/Pvu II fragment of pGA714. This fragment contains all of the promoter regions needed for the expression of Kex2p in yeast but only encodes a truncated Kex2p (Kex2'p) of 614 amino acids in length. The Kex2'p still contains the predicted signal sequence and the domain of homology to subtilisin, but the Ser/Thr rich domains and the transmembrane domains have been removed.

A



The Kex2 polypeptide

B



The truncated Kex2 polypeptide (Kex2'p)

Fig. 4.2 Construction of the intermediate plasmid pKkex'

For this construction the source of Kex2p-coding DNA was pGA714. The Eco RI/Pvu II fragment from pGA714 was purified: this contains the promoter regions needed for the expression of Kex2p in yeast and the coding sequence for the first 614 amino acids of Kex2p. Plasmid pK19 was digested with Bam HI and the ends were made blunt by filling in. This DNA was then digested with EcoRI. The Eco RI/Pvu II fragment from pGA714 was ligated with the Bam HI (filled in) Eco RI digested pK19 and transformed into NM522. The resulting plasmid was named pKkex'. The Pvu II site ligated to the Bam HI filled in site re-creates the BamHI site. Plasmid pKkex' contains the coding sequence for a truncated Kex2p along with the promoter regions needed for its expression in yeast. The re-created Bam HI site is of use for cloning oligonucleotides into.

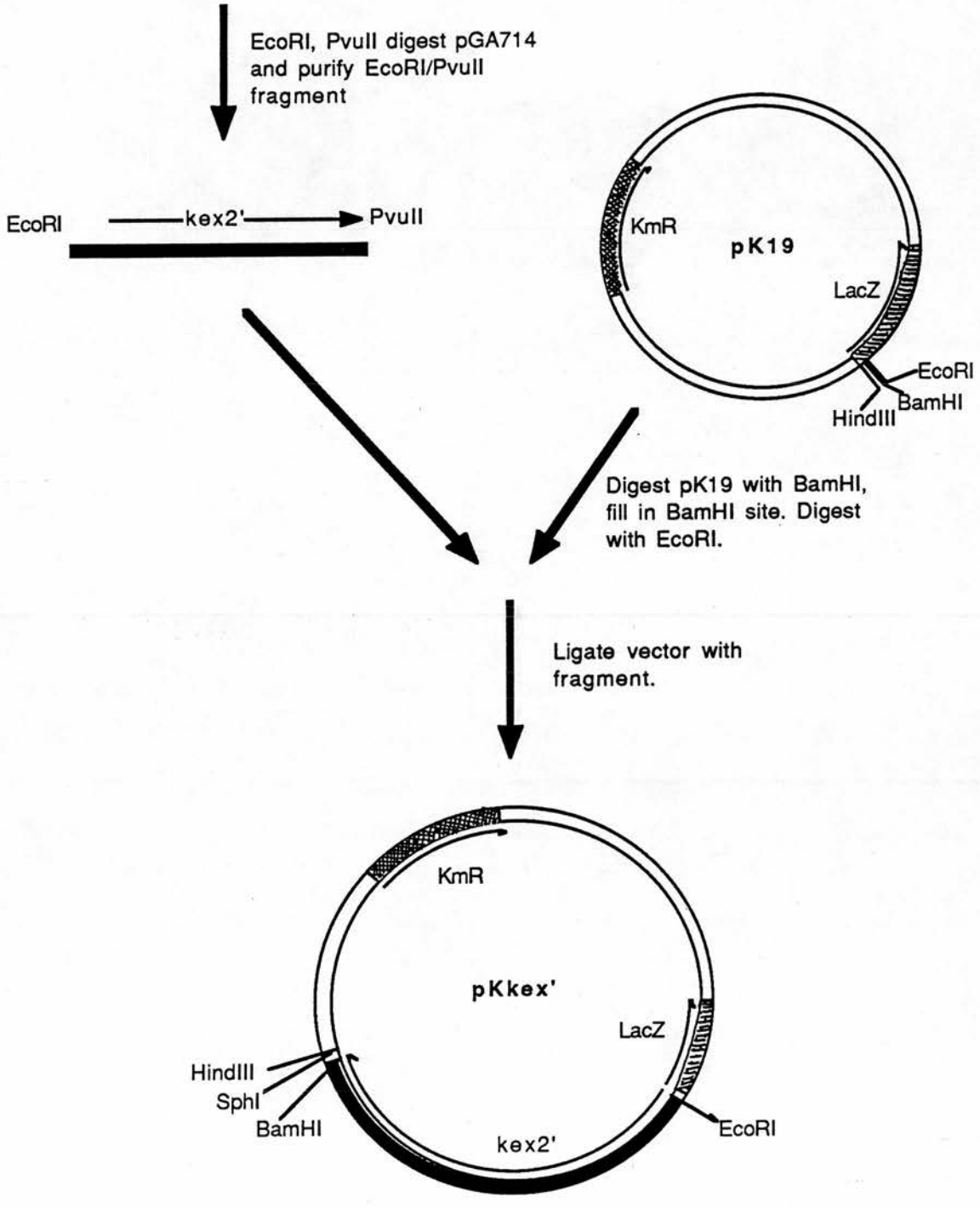
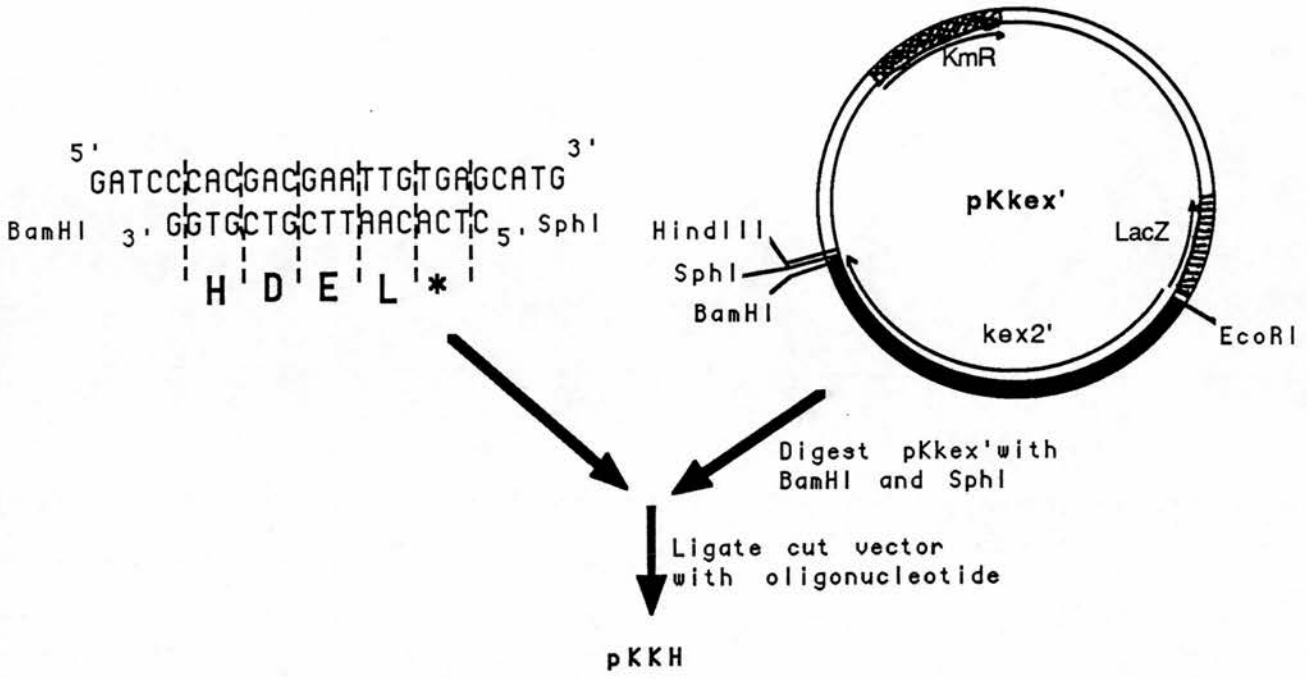
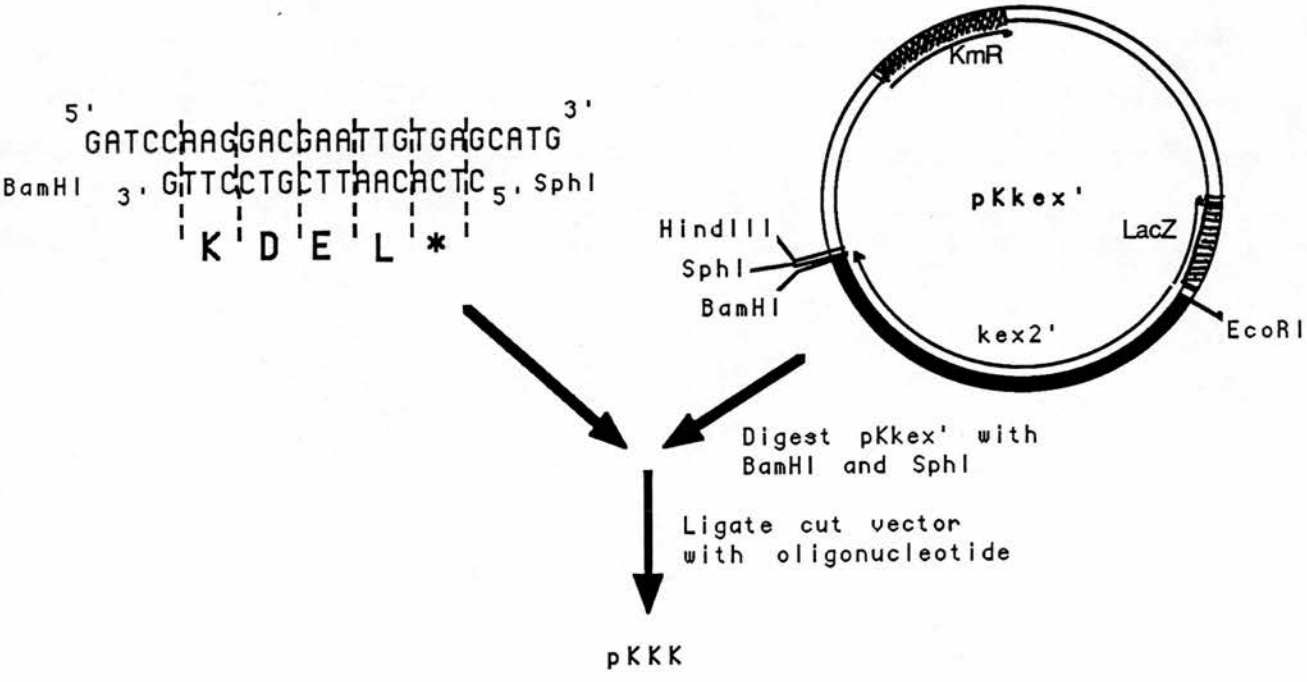

EcoRI, PvuII digest pGA714
and purify EcoRI/PvuII
fragment
EcoRI
kex2'
PvuII
KmR
pK19
LacZ
EcoRI
BamHI
HindIII
Digest pK19 with BamHI,
fill in BamHI site. Digest
with EcoRI.
Ligate vector with
fragment.
KmR
pKkex'
LacZ
HindIII
SphI
BamHI
kex2'
EcoRI

Fig. 4.3 Attachment of the C-terminal tetrapeptides HDEL and KDEL onto Kex2'p

Attachment of the HDEL and KDEL sequences to the C-terminus of Kex2'p was achieved by cloning oligonucleotides into plasmid pKkex'. The HDEL and KDEL oligonucleotides were designed to have Bam HI and Sph I compatible ends. Plasmid pKkex' was digested with Bam HI and Sph I, ligated with the HDEL or KDEL oligonucleotide and then transformed into NM522. Cloning of the oligonucleotides into the Bam HI/Sph I sites of pKkex' re-creates Bam HI and Sph I sites and removes a Sal I site (the Sal I site is in the pK19 polylinker between the Bam HI and Sph I sites: this region of the polylinker is removed when the vector is digested with Bam HI and Sph I). Recombinant plasmids were screened for (by restriction digest analysis) that had lost the Sal I site. The resulting plasmids pKKH and pKKK contain the HDEL and KDEL coding sequences in frame with the Kex2p coding sequence. Thus plasmid pKKH and pKKK encode Kex2' polypeptides with HDEL and KDEL (Kex2'-HDEL and Kex2'-KDEL) at their extreme C-termini.


5' GATCCACGACGAATTGTGAGCATG 3'
BamHI 3' GGTGCTGCTTAACACTC 5' SphI
H D E L *
KmR
pKkex'
LacZ
HindIII
SphI
BamHI
kex2'
EcoRI
Digest pKkex'with BamHI and SphI
Ligate cut vector with oligonucleotide
pKKH


5' GATCCAAGACGAATTGTGAGCATG 3'
BamHI 3' GTTCCTGCTTAACACTC 5' SphI
K D E L *
KmR
pKkex'
LacZ
HindIII
SphI
BamHI
kex2'
EcoRI
Digest pKkex' with BamHI and SphI
Ligate cut vector with oligonucleotide
pKKK

with a Bam HI compatable end and an Sph I compatable end so that they could be cloned into the Bam HI, Sph I sites of pKkex' to create pKKH or pKKK (fig. 4.3). The constructs pKKH and pKKK were confirmed to be correct by DNA sequencing (see materials and methods) of suitable fragments from these plasmids cloned into M13mp18 (fig. 4.4).

At this stage oligonucleotides coding for the HDEL and KDEL tetrapeptides had been cloned in frame with the coding sequence for Kex2'p at its C-terminus. However the plasmids pKKH and pKKK cannot be used to express the Kex2' polypeptides with the different C-teminal sequences in yeast. Therefore it was necessary to move the *kex2'* genes possessing the HDEL and KDEL coding oligonucleotides into vectors that could be transformed into yeast. The Eco RI/Sph I fragments from pKKH and pKKK contain the Kex2 promoter region, the coding region for the first 614 amino acids of Kex2p and the oligonucleotides coding for HDEL or KDEL respectively. The Eco RI/Sph I fragments from pKKH and pKKK were cloned into the Eco RI, Sph I sites of two different plasmids YEplac112 and YCplac22 (fig. 4.5) (Gietz and Sugino 1988). Both YEplac112 and YCplac22 are useful shuttle vectors as they contain the pUC19 polylinker in which all the restriction sites can be used because any duplicate sites in the vectors have been mutated out. Both vectors possess the ampicillin resistance gene as a selective marker in *E.coli*. The screening for foreign DNA cloned into YEplac112 and YCplac22 polylinkers is made easier as the blue/white colour test on X-Gal plates can be used. YEplac112 is an episomal plasmid (multicopy in yeast) and possesses the *TRP1* gene as a selectable marker in yeast. YCplac22 is a centromere plasmid (essentially single copy in yeast) and also possesses the *TRP1* gene as a selectable marker.

The plasmids resulting from the cloning of the Eco RI/Sph I fragments from pKKH and pKKK into YEplac112 were called YEpKH and YEpKK respectively (fig. 4.5). The plasmids resulting from the cloning of the Eco RI/Sph I fragments from pKKH and pKKK into YCp lac22 were called YCpKH and YCpKK respectively (fig. 4.5). It was also decided to construct vectors from which Kex2'p with no specific C-terminal retention signals could be expressed in yeast. These vectors were constructed by cloning a stop oligonucleotide into the Bam HI sites of YEpKH and YCpKH to create plasmids called YEpKS and YCpKS respectively (fig. 4.6).

Fig. 4.4 DNA sequence analysis of the Kex2'-HDEL and Kex2'-KDEL constructs

To check whether plasmids pKKH and pKKK were the correct constructs it was decided to partially sequence the constructs in order to see if the HDEL and KDEL oligonucleotides had been cloned into pKkex' correctly. The Bgl II/Hind III fragments from pKKH and pKKK were cloned into the Bam HI/Hind III sites of M13mp18 (Bgl II creates ends compatible with Bam HI). These M13 recombinants were sequenced as descibed in Materials and Methods. The sequences (A) and (B) confirm that the correct oligonucleotides have been cloned in at the correct position. Thus pKKK and pKKH contain DNA coding for Kex'-HDEL and Kex2'-KDEL polypeptides respectively.

A T C G
E P G S K D E L *
CAACTTGGTCCTAGGTTCCTGCTTAACACTCGTACG
A T C G
E P G S H D E L *
CAACTTGGTCCTAGGGTGCTGCTTAACACTCGTACG

Fig. 4.5 Construction of vectors for the expression of the Kex2'-HDEL and Kex2'-KDEL polypeptides in yeast

Plasmids pKKH and pKKK were the sources of DNA encoding for Kex2'-HDEL and Kex2'-KDEL respectively. The Eco RI/Sph I fragments from pKKH or pKKK were ligated with EcoRI/SphI digested YCplac22 or Eco RI/Bam HI digested YEplac112 and transformed into NM522. Recombinant plasmids were screened for by a blue/white colour test on X-Gal plates. The resulting plasmids were called YCpKH, YCpKK, YEpKH and YEpKK respectively. YCpKH and YEpKH contain the promoter region of Kex2 and when transformed into yeast should express the Kex2'-HDEL polypeptide. YCpKK and YEpKK contain the promoter region of Kex2 and when transformed into yeast should express the Kex2'-KDEL polypeptide. Plasmids YCpKH and YCpKK can be transformed into yeast and should be present in a single copy per cell. Plasmids YEpKH and YEpKK can be transformed into yeast and should be present in multi-copy.

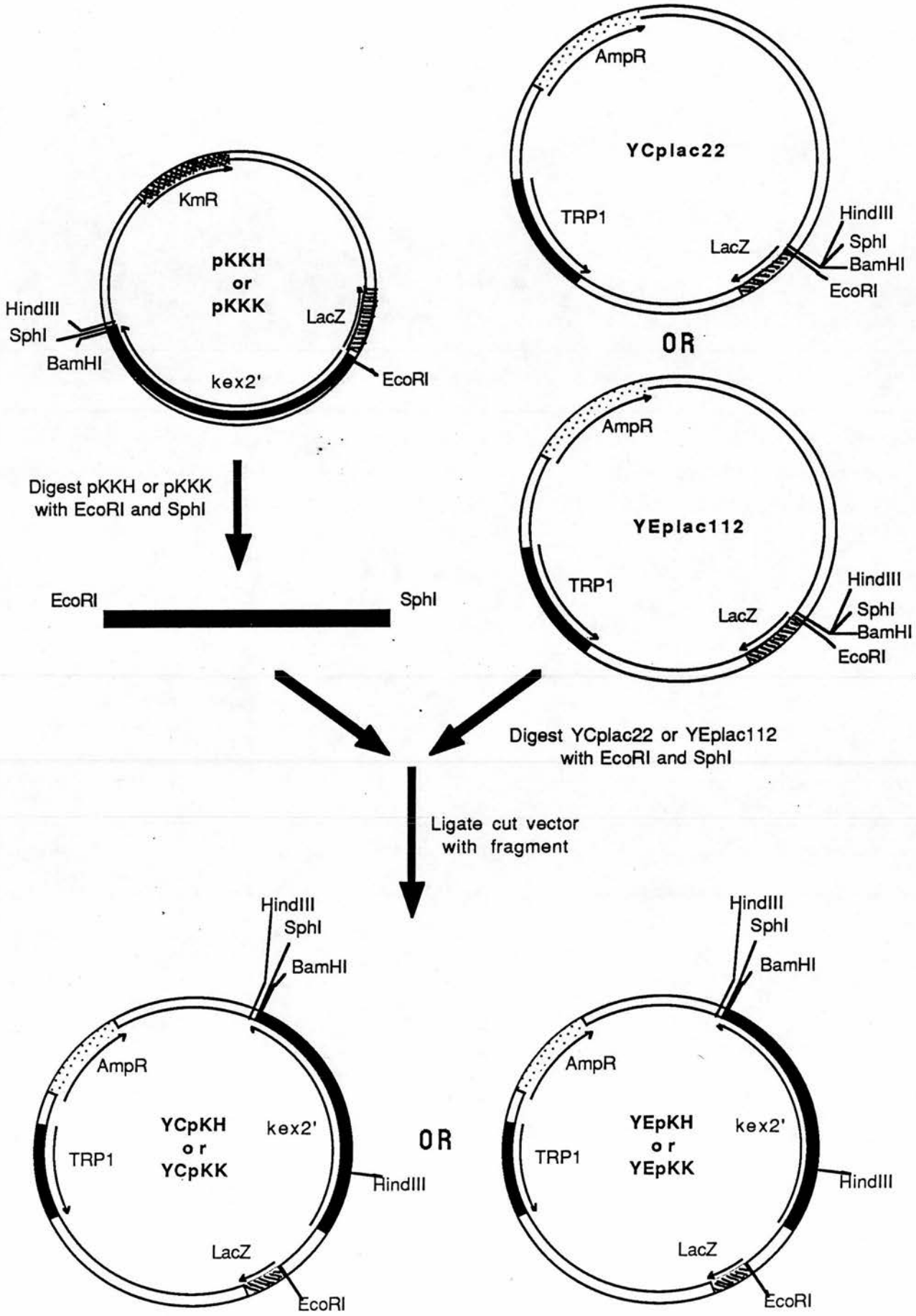

AmpR
YCplac22
TRP1
LacZ
HindIII
SphI
BamHI
EcoRI
OR
KmR
pKKH
or
pKKK
LacZ
HindIII
SphI
BamHI
kex2'
EcoRI
AmpR
YEplac112
TRP1
LacZ
HindIII
SphI
BamHI
EcoRI
Digest pKKH or pKKK
with EcoRI and SphI
EcoRI
SphI
Digest YCplac22 or YEplac112
with EcoRI and SphI
Ligate cut vector
with fragment
HindIII
SphI
BamHI
AmpR
YCpKH
or
YCpKK
kex2'
TRP1
HindIII
LacZ
EcoRI
OR
HindIII
SphI
BamHI
AmpR
YEpKH
or
YEpKK
kex2'
TRP1
HindIII
LacZ
EcoRI

Fig. 4.6 Construction of vectors for the expression of Kex2'-S in yeast

To construct plasmids for the expression of a Kex2'p (Kex2'-S) with no additional amino acids at the C-terminus an oligonucleotide was designed that could be cloned into the Bam HI site of YCpKH and YEpKH. The oligonucleotide has stop codons in all three of its reading frames but in the case of this cloning the first stop codon is in frame with the Kex2 reading frame. The oligonucleotide also has a Nhe I restriction site in it which makes screening for insertion of the oligonucleotide possible by restriction analysis. Plasmid YCpKH or YEpKH were digested with Bam HI, ligated with the stop oligonucleotide and then transformed into NM522. Recombinant plasmids were screened for by restriction analysis for the introduction of an extra Nhe I site. The resulting plasmids were called YCpKS and YEpKS respectively. Plasmid YCpKS and YEpKS encode Kex2'-S that has no additional amino acids at the C-terminus along with the Kex2 promoter regions needed for its expression in yeast. Plasmid YCpKS and YEpKS can be transformed into yeast and should be present in a single copy and multi-copy respectively.

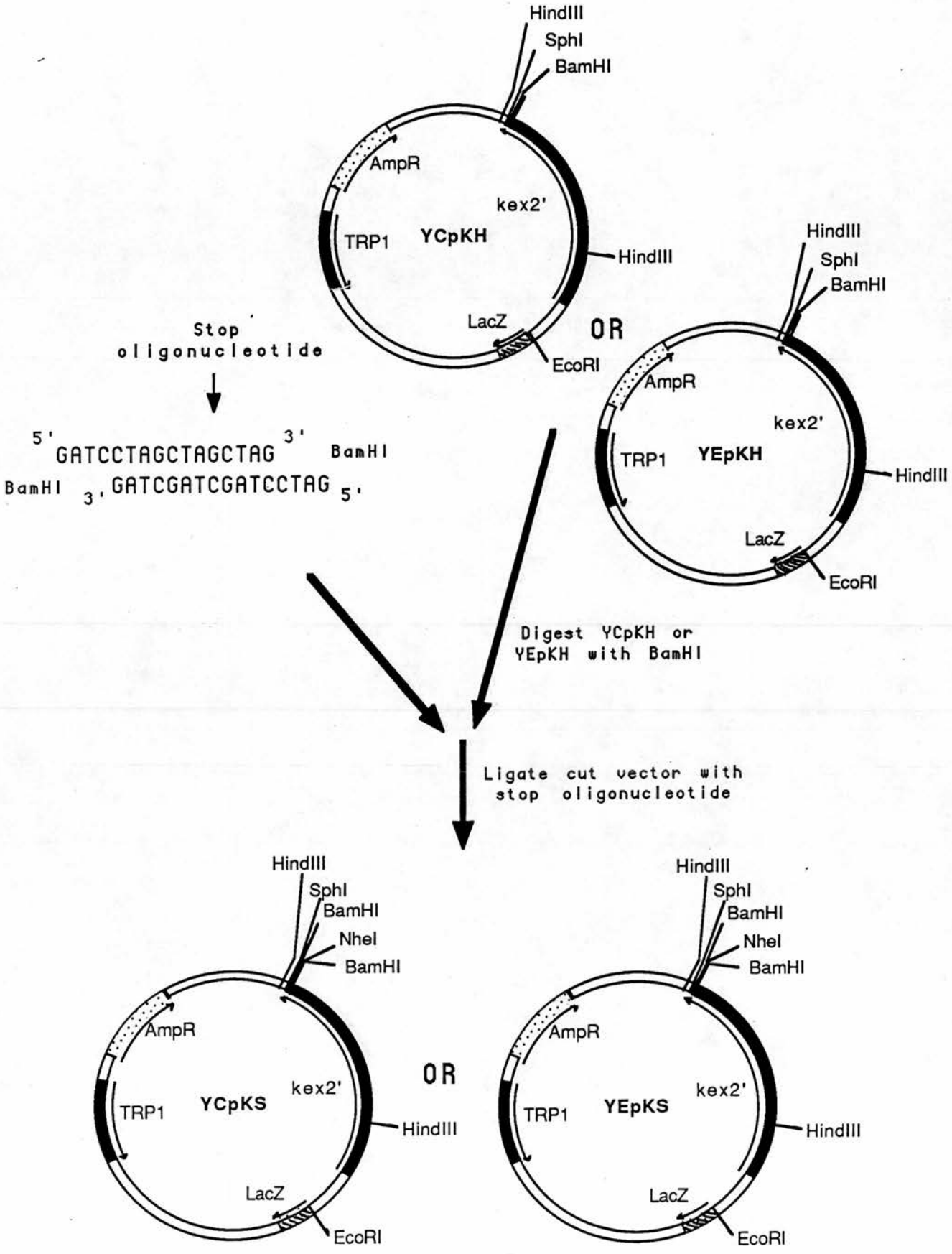

HindIII
SphI
BamHI
AmpR
kex2'
TRP1
YCpKH
HindIII
LacZ
EcoRI
OR
HindIII
SphI
BamHI
AmpR
kex2'
TRP1
YEpKH
HindIII
LacZ
EcoRI
Stop
oligonucleotide
5' GATCCTAGCTAGCTAG 3' BamHI
BamHI 3' GATCGATCGATCCTAG 5'
Digest YCpKH or
YEpKH with BamHI
Ligate cut vector with
stop oligonucleotide
HindIII
SphI
BamHI
NheI
BamHI
AmpR
TRP1
YCpKS
kex2'
HindIII
LacZ
EcoRI
OR
HindIII
SphI
BamHI
NheI
BamHI
AmpR
TRP1
YEpKS
kex2'
HindIII
LacZ
EcoRI

Table 4.1 Plasmid constructs for the expression of truncated Kex2 polypeptides (Kex2'p s) with different C-termini in yeast

Name of plasmid	C-terminus of Kex2'p expressed	Name of Kex2'p expressed
YCpKH (single copy in yeast)	PGSHDEL*	Kex2'-HDEL
YCpKK (single copy in yeast)	PGSKDEL*	Kex2'-KDEL
YCpKS (single copy in yeast)	PGS*	Kex2'-S
YEpKH (multi-copy in yeast)	PGSHDEL*	Kex2'-HDEL
YEpKK (multi-copy in yeast)	PGSKDEL*	Kex2'-KDEL
YEpKS (multi-copy in yeast)	PGS*	Kex2'-S

The truncated Kex2 polypeptides with the different C-termini, expressed from the different plasmids will be referred to in future chapters by the nomenclature shown in this table.

4.3 Summary

In this chapter I have described the construction of six vectors (see table 4.1) that can be used to express truncated Kex2 polypeptides with the different C-termini in yeast. Experiments to look at the retention of the different constructs, their localisation in the cell and their effect on the processing and secretion of secretory proteins in yeast will be described in following chapters.

Chapter five.

In vitro and *in vivo* assays for Kex2 activity in strains of yeast expressing the truncated Kex2 polypeptides possessing the different ER retention signals.

5.0 Introduction

In the previous chapter the construction of vectors for the expression in yeast of truncated Kex2p's with different C-termini in was described. In this chapter I will describe *in vivo* and *in vitro* methods for comparing the properties of the different constructs with respect to their ability to be retained within the cell and their ability to process proteins in yeast. All of the yeast plasmids (episomal and centromere) containing the various Kex2 constructs carried the *TRP1* gene as a selective marker. It was therefore necessary to transform these plasmids into a strain of yeast lacking a functional *TRP1* gene. Also the strain of yeast to be transformed had to lack a functional *KEX2* gene in order that the properties of the truncated Kex2p's could be assessed without interference from the native *KEX2* gene.

5.1 The disruption of the functional *KEX2* gene in *Saccharomyces cerevisiae* strain JRY188.

JRY188 is a strain of yeast regularly used in our laboratory (For genotype see strain list table 2.1). The *KEX2* gene of JRY188 was disrupted by a gene replacement method in which the chromosomal *KEX2* gene was replaced by a gene that could easily be selected for (in this case the *LEU2* or *URA3* genes). The plasmids pGA1070 and pGA1320 (supplied by Dr Gustav Ammerer) were digested with BamHI and SphI, and fragments of DNA containing the majority of the *KEX2* gene replaced with the *LEU2* (kex2::LEU2) or *URA3* (kex2::URA3) genes respectively were purified (figs 5.1 and 5.2). The fragments still contained the flanking regions of the *KEX2* gene and some of the C-terminal coding sequences of the *KEX2* gene. These purified fragments were introduced into JRY188 by transformation with selection for Leu^+ (when transformed with kex2::LEU2) or Ura^+ (when transformed with kex2::URA3) transformants (untransformed JRY188 is Leu^-,Ura^-). Such Leu^+ or Ura^+ transformants are expected to have the chromosomal copy of *KEX2* replaced with the kex2::LEU2 or kex2::URA3 gene disruptions respec tively, by homologous recombination of the flanking regions (fig. 5.1 and 5.2). Transformants were checked for the replacement of the *KEX2* gene with the disrupted *KEX2* genes by Southern analysis of the chromosomal DNA of the transformants (fig. 5.3). The Southern confirmed replacement of the *KEX2* gene with the disrupted *KEX2* genes. The resulting strains were named PWYS2 (JRY188 kex2::LEU2) and PWYS3

Fig5.1 Replacement of the chromosomal copy of *KEX2* in JRY188 with a non functional disrupted *KEX2* gene (kex2::LEU2).

The Bam HI/Sph I fragment of pGA1070 (supplied by Dr. G. Ammerer) has most of the *KEX2* gene replaced with the *LEU2* gene (*kex2::LEU2*), but the flanking regions are intact. Yeast strain JRY188 was transformed with the *kex2::LEU2* disruption from pGA1070 with selection for Leu^+ transformants. Homologous recombination of the regions flanking the *kex2::LEU2* disruption in the Bam HI/Sph I fragment and the regions flanking the chromosomal copy of the *KEX2* gene results in the replacement of the chromosomal copy of the *KEX2* gene with the *kex2::LEU2* disruption. The resulting strain PWYS2 does not contain a functional KEX2 gene. The restriction sites shown are:- Bam HI (B), Bgl II (Bg), Eco RI (E), Hind III (H), Sph I (S) and Pvu II (P).

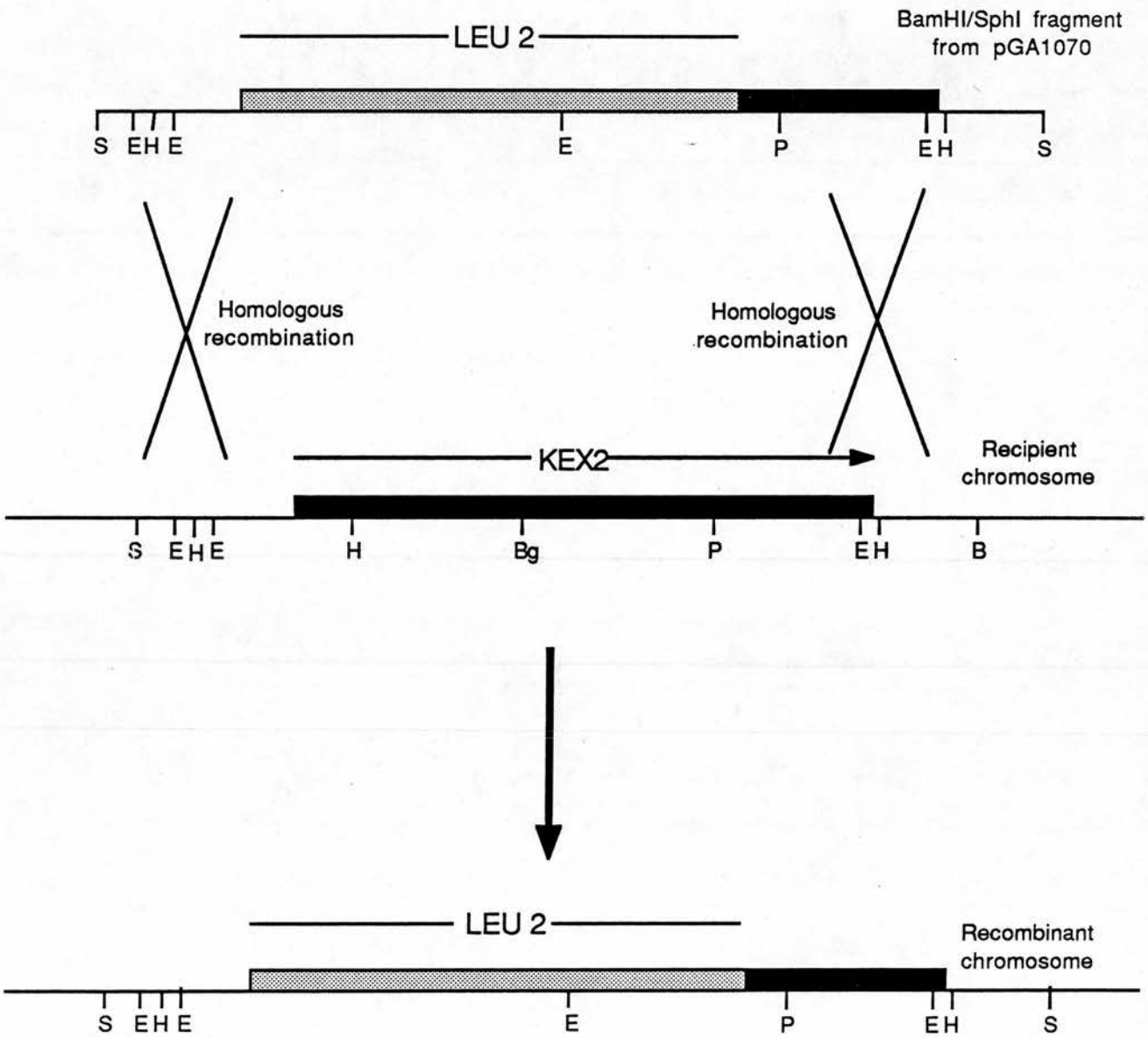

LEU 2
BamHI/SphI fragment
from pGA1070
S EH E
E
P
EH
S
Homologous
recombination
Homologous
recombination
KEX2
Recipient
chromosome
S EH E
H
Bg
P
EH
B
LEU 2
Recombinant
chromosome
S EH E
E
P
EH
S

Fig 5.2 Replacement of the chromosomal copy of *KEX2* in JRY188 with a non functional disrupted *KEX2* gene (*kex2::URA3*).

The Bam HI/Sph I fragment of pGA1320 (supplied by Dr. G. Ammerer) has most of the *KEX2* gene replaced with the *URA3* gene (*kex2::URA3*), but the flanking regions are intact. Yeast strain JRY188 was transformed with the *kex2::URA3* disruption from pGA1070 with selection for Ura$^+$ transformants. Homologous recombination of the regions flanking the *kex2::URA3* disruption in the Bam HI/Sph I fragment and the regions flanking the chromosomal copy of the *KEX2* gene results in the replacement of the chromosomal copy of the *KEX2* gene with the *kex2::URA3* disruption. The resulting strain PWYS3 does not contain a functional KEX2 gene. The restriction sites shown are:- Bam HI (B), Bgl II (Bg), Eco RI (E), Hind III (H), Sph I (S) and Pvu II (P).

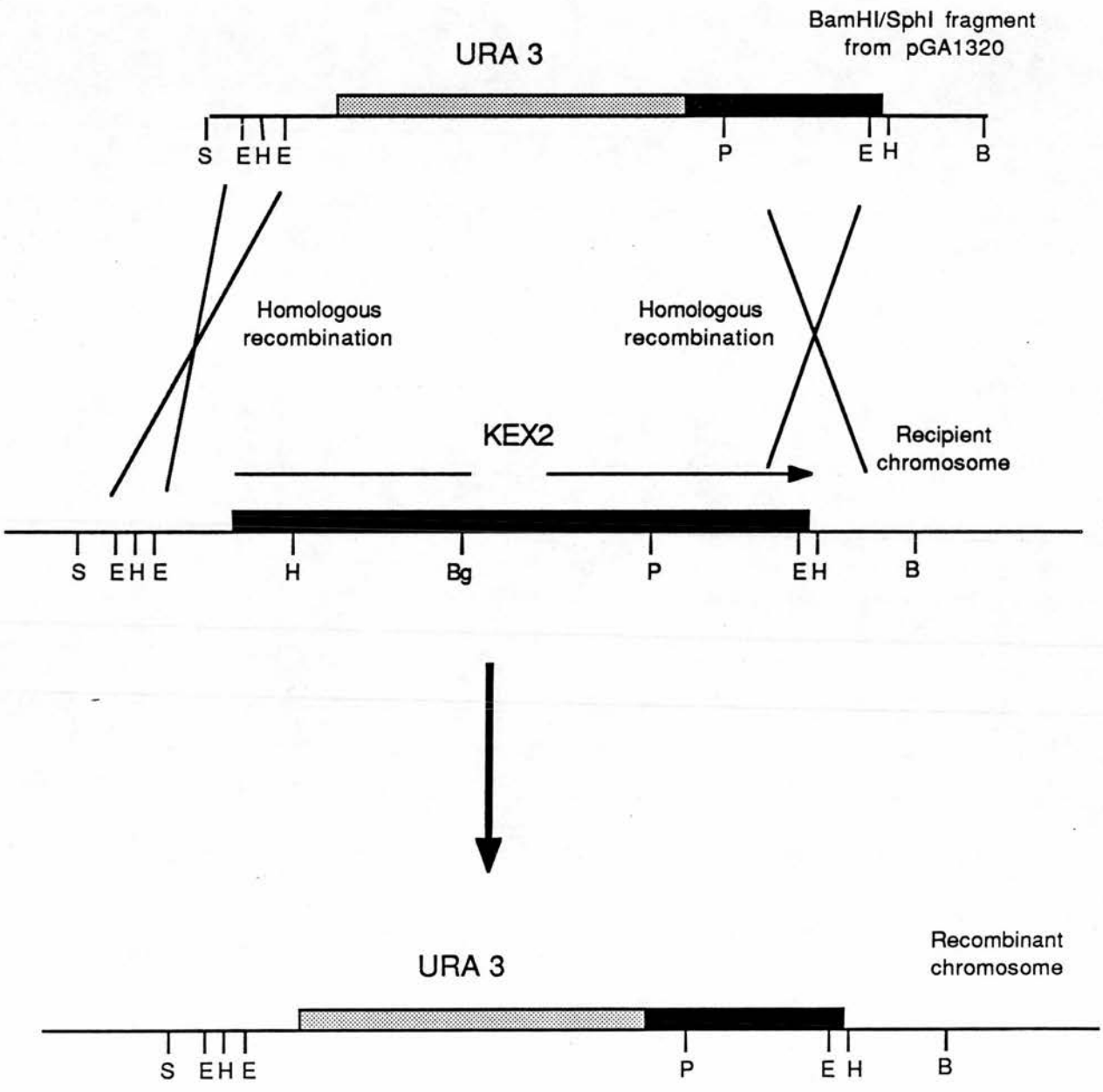

BamHI/SphI fragment
from pGA1320
URA 3
S EHE
P
EH
B
Homologous
recombination
Homologous
recombination
KEX2
Recipient
chromosome
S EHE
H
Bg
P
EH
B
Recombinant
chromosome
URA 3
S EHE
P
EH
B

Fig. 5.3 Southern analysis of JRY188 and JRY188 transformants.

Total DNA from JRY166 and JRY188 transformed with the *kex2::LEU2* or *kex2::URA3* disruption fragments were digested with Eco RI and electrophoresed on a 0.8% agarose gel. The DNA was blotted onto nitrocellulose. Southern analysis was performed using a radiolabeled DNA probe. The DNA fragment used as the probe is shown in the figure and consists of a 3.6 kb Eco RI fragment of the *KEX2* gene (purified from plasmid pGA714, supplied by Dr. G Ammerer). The lanes of the autoradiograph are as follows:-

1. JRY188

2. JRY188 transformed with *kex2::LEU2* disruption fragment (n^o1).

3. JRY188 transformed with *kex2::LEU2* disruption fragment (n^o2).

4. JRY188 transformed with *kex2::LEU2* disruption fragment (n^o3).

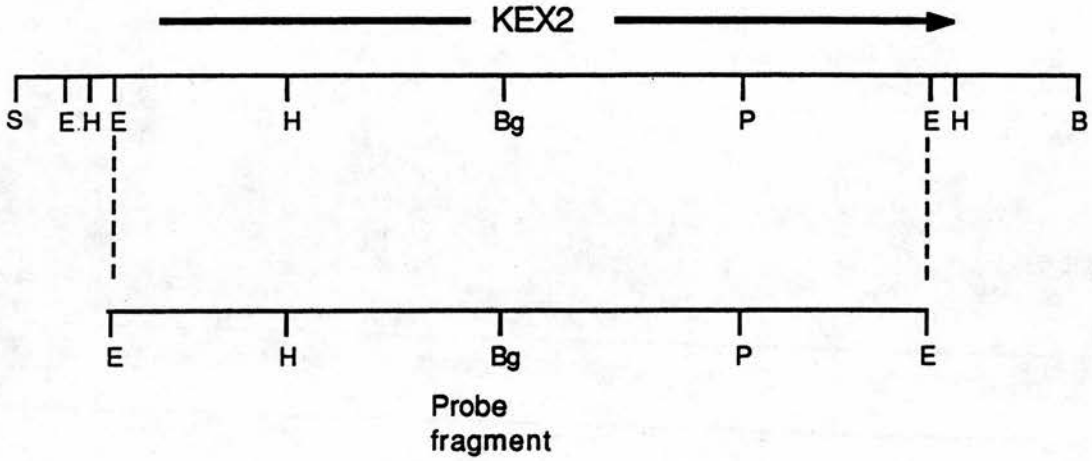
5. JRY188 transformed with *kex2::LEU2* disruption fragment (n^o4).

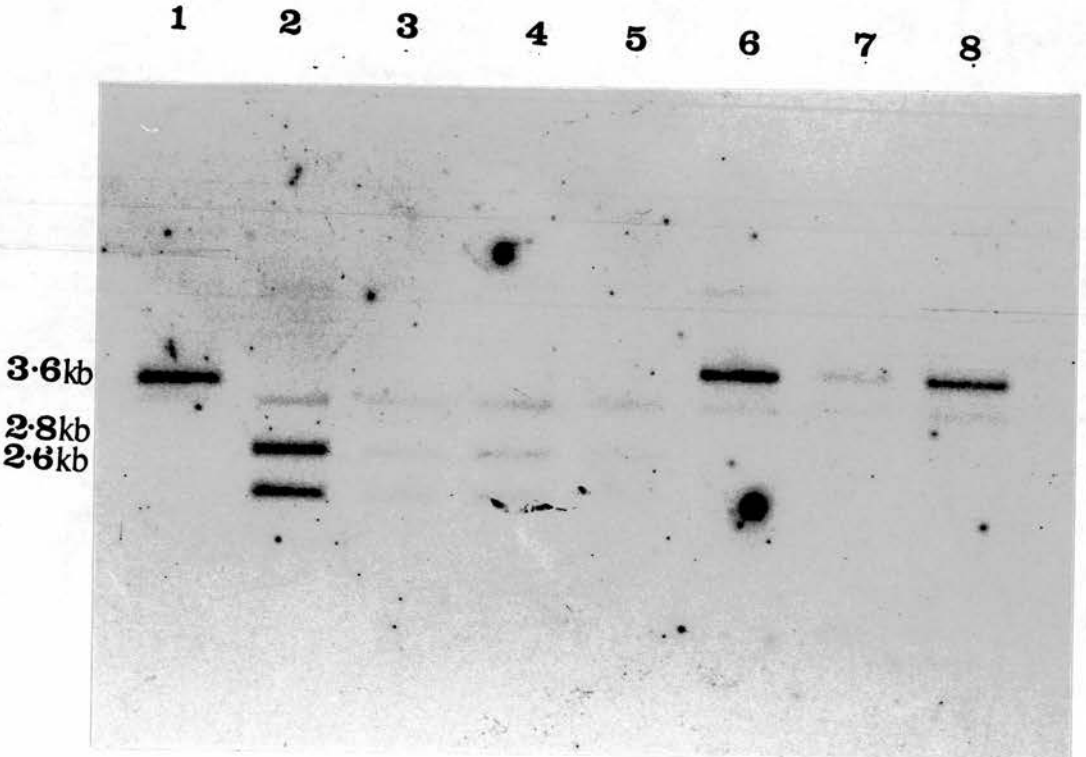
6. JRY188 transformed with *kex2::URA3* disruption fragment (n^o1).

7. JRY188 transformed with *kex2::URA3* disruption fragment (n^o2).

8. JRY188 transformed with *kex2::URA3* disruption fragment (n^o3).

The 3.6 kb band on the autoradiograph is indicative of the *KEX2* gene. The 2.8 kb and 2.6 kb bands are indicative of the *kex2::LEU2* disruption fragment being present (when *kex2::LEU2* is digested with Eco RI, two fragments of 2.8 kb and 2.6 kb are released; Both of them are recognised by the *KEX2* probe). The 3.7 kb band is indicative of the kex2::URA3 disruption fragment being present.


KEX2
S
E
H
E
H
Bg
P
E
H
B
E
H
Bg
P
E
Probe
fragment


1
2
3
4
5
6
7
8
3·6kb
2·8kb
2·6kb

(JRY188 kex2::URA3). PWYS3 was the strain which was to be transformed with the following plasmids:- YCpKH, YCpKK, YCpKS, YEpKH, YEpKK and YEpKS. These plasmids were introduced into PWYS3 with selection for Trp^+ transformants.

5.2 Assay for Kex2 activity in vitro

The enzymic activity of Kex2p can be assayed directly by measuring the amount of a fluorescent molecule (AMC) released as a result of cleavage of a synthetic peptide (bQRR-MCA) at the C-teminal side of the pair of basic amino acids (see Materials and Methods). It was decided to look at the intracellular and extracellular Kex2 activities in the different transformed yeast strains to determine whether the different C-termini on the truncated Kex2p's had any effect on their retention within the cell. If there is specific retention within the cell due to the different C-termini, comparison of the relative amounts of intracellular and extracellular activity in the different strains should give an indication of this.

The yeast strains to be assayed for intracellular and extracellular Kex2 activities were grown in their respective selective media for 16 hours. The optical densities of the different cutures were measured at 600nm. The cells were harvested and the supernatants were kept for the assay of extracellular Kex2 activities. The cells that had been harvested were washed and the cells were permeabilised by treatment with Brij 58 (see Materials and Methods). These Brij-permeabilised cells were the source of intracellular Kex2p for the different strains. The assays for Kex2 activity were carried out on 5µl samples of each of the Brij-permeabilised cells and 5µl samples of the supernatants to give a measure of the relative amounts of intracellular and extracellular Kex2 activities in the different strains. The results were corrected for cell density as the different strains had grown to different densities when grown in their respective selective medias for 16 hours. The results of the assays are represented in Table 5.1 and fig. 5.4.

The results of the direct assay for intracellular and extracellular Kex2 activity in the different strains of yeast show a number of interesting features. As expected there is hardly any detectable Kex2 activity in PWYS3. The small amount of

Table 5.1 The intracellular and extracellular Kex2 activities of yeast strains expressing the different Kex2' polypeptides

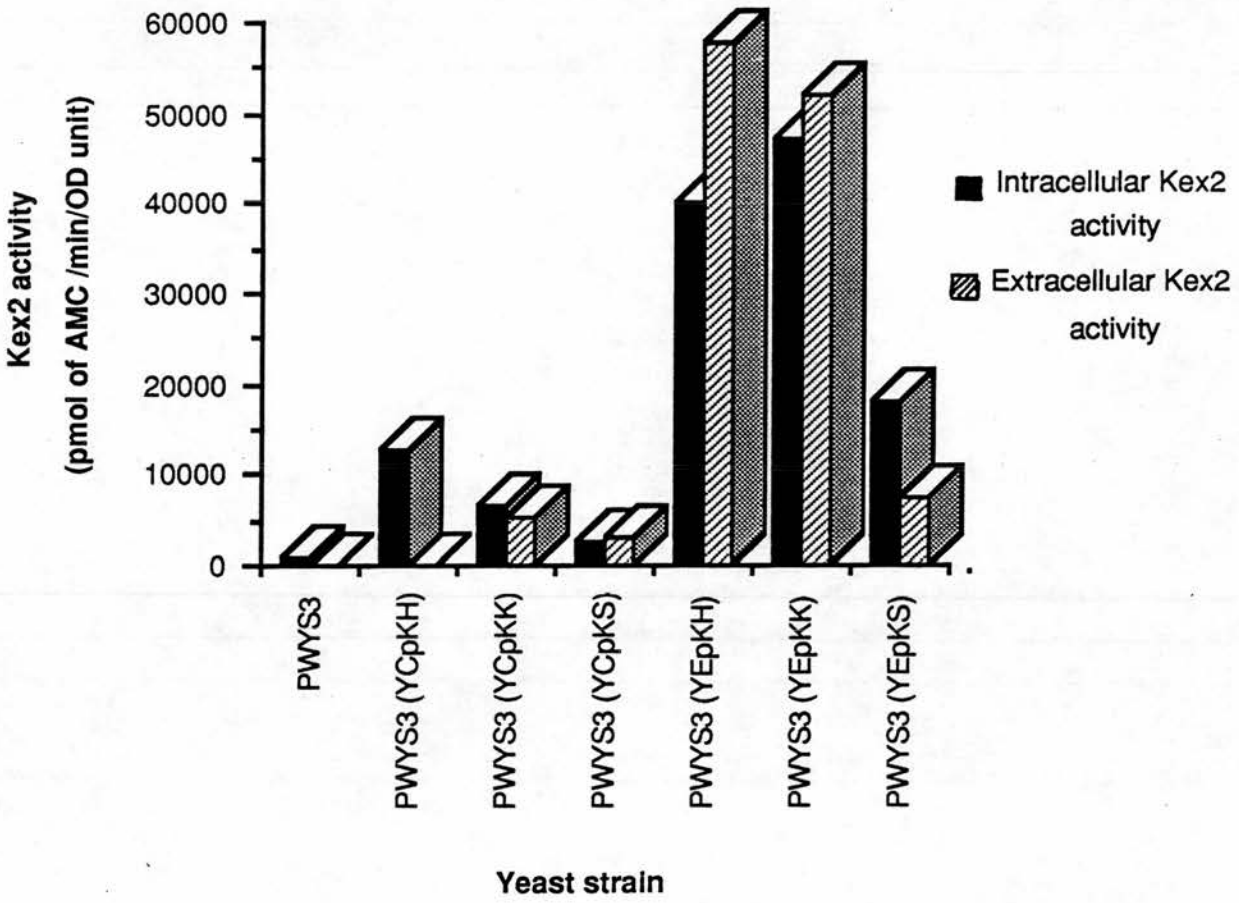
Yeast strain	Intracellular Kex2 activity	Extracellular Kex2 activity
PWYS3	7.2×10^2 (-)	0 (-)
PWYS3 (YCpKH)	1.3×10^4 (1.6×10^3)	0 (-)
PWYS3 (YCpKK)	6.7×10^3 (2.2×10^3)	5.1×10^3 (4.3×10^2)
PWYS3 (YCpKS)	2.5×10^3 (7.0×10^2)	3.0×10^3 (3.4×10^2)
PWYS3 (YEpKH)	4.0×10^4 (2.1×10^3)	5.8×10^4 (4.6×10^2)
PWYS3 (YEpKK)	4.7×10^4 (2.5×10^3)	5.2×10^4 (3.2×10^3)
PWYS3 (YEpKS)	1.8×10^4 (3.6×10^3)	7.7×10^3 (5.2×10^2)

The Kex2 activities are in picomoles of AMC released from bQRR-MCA per minute, per OD unit of cells. The results are an average of three separate Kex2 assays carried out in standard conditions (see Materials and Methods), and the standard deviations from the mean are shown in brackets. Intracellular Kex2 activities were obtained by assaying Brij-permabilised cells (see Materials and Methods) of cells from each strain. Extracellular activities were obtained by assaying the supernatants of each strain grown in selective media.

Fig 5.4. **A graph showing the intracellular and extracellular Kex2 activities of yeast strains expessing the different Kex2' polyeptides (I).**

The *in vitro* intracellular and extracellular Kex2 activities (shown in Table 5.1) for the strains expressing the different Kex2' polypeptides are plotted. The Kex2 activities are in picomoles of AMC released from the synthetic peptide bQRR-MCA per minute, per OD unit of cells (pmol of AMC/min/OD unit).

There is some variability in the activities of individual strains in separate experiments, but the general trends in activities between strains is still observed.


Kex2 activity
(pmol of AMC /min/OD unit)
60000
50000
40000
30000
20000
10000
0
PWYS3
PWYS3 (YCpKH)
PWYS3 (YCpKK)
PWYS3 (YCpKS)
PWYS3 (YEpKH)
PWYS3 (YEpKK)
PWYS3 (YEpKS)
Yeast strain
Intracellular Kex2 activity
Extracellular Kex2 activity

intracellular activity detected may be due to other proteases in the cell cleaving the synthetic peptide non-specifically. In general the overall Kex2 activities in strains bearing the episomal plasmids is higher than those bearing the centromere plasmids which is consistent with the episomal plasmids being present in higher number of copies per cell than the centromere plasmids, which are single copy per cell. The strains bearing the centromere plasmids expressing the different Kex2'p's show some interesting differences. The strain expressing the Kex2'-HDEL has the highest internal Kex2 activity but there is no detectable external Kex2 activity. The strain expressing Kex2'-KDEL contains the next highest level of intracellular Kex2 activity but it also appears to secrete Kex2p as there is extracellular Kex2 activity detectable in the medium. The strain expressing the Kex2'-S has the least amount of intracellular Kex2 activity but also the least amount of total Kex2 activity. These results suggest that the HDEL sequence at the C-terminus is preventing the Kex2p from being secreted as all of the detectable Kex2 activity is intracellular. The KDEL sequence may have some ability to prevent secretion of Kex2p from the cell as there is more intracellular Kex2 activity than when the stop codon is at the C-terminus of Kex2'p which should not have any specific retension sequences in it. The reason for the different total Kex2 activities (ie the sum of intracellular and extracellular activities) in the different strains is difficult to explain as all the strains should express Kex2' polypeptides at the same level even though the amounts retained may be different. We have assumed that the addition of different C-temini will not affect the specific activity of the truncated polypeptides, although this assumption has not been tested. If the assumption is not correct the different total activities could be due to differing catalytic activities of the different Kex2' constructs. Another possibility is that the different Kex2' constructs are differentially degraded or denatured either intracellularly or extracellularly.

As has been mentioned already the cells harbouring the episomal plasmids all have relatively high levels of intracellular and extracellular Kex2 activities in comparison to the centromere plasmid bearing strains. Even the strain expressing Kex2'-HDEL has a high level of extracellular Kex2 activity suggesting that Kex2'-HDEL is not efficiently retained within the cell. As with the centromere plasmids the strain expressing Kex2'-S has the lowest total Kex2 activity which is again difficult to explain. A reason for the high extracellular Kex2 activity in all of the strains including the strain expressing Kex2'-HDEL could possibly be that the

retention system is being saturated, which has been shown to be possible in yeast (Pelham *et al* 1988, Dean and Pelham 1990). However, as wild type Kex2p is very inabundant in yeast when expressed from its own promoter (Fuller *et al* 1989) and all of the Kex2' polypeptides are expressed from this same promoter we would not expect the levels of the Kex2' polypeptides being produced, even from multicopy plasmids to be enough to saturate a retention system that can supposedly retain abundant proteins such as BiP and PDI in yeast. Another explanation could be that the sequences attached to the C-termini of the different Kex2' polypeptides are not efficiently recognised by the retention system because of the way in which the proteins are folded. This does not explain why the Kex2'-HDEL is apparently efficiently retained in the cell when expressed at lower levels in cells containing the centromere plasmids.

5.3 Assay for Kex2 activity *in vivo*

The Kex2' polypeptides with the different C-termini all appear to be active, as they cleave the synthetic peptide bQRR-MCA in the *in vitro* assay for Kex2 activity. It still remained to be seen however, whether these Kex2' polypeptides could process proteins *in vivo* and if so, what might be the effect the different C-termini upon this processing. It was decided to look initially at the processing of the yeast mating pheromone α factor in strains expressing the different Kex2' polypeptides. The way in which α factor is normally processed and the involvement of wild type Kex2p in this processing is described in the Introduction. We wanted to determine how efficiently strains transformed with the plasmids for expression of the Kex2' polypeptides processed and secreted α factor, and if the C-terminal sequences affected this efficiency.

5.4 The assay for secreted α factor

It is possible to detect the presence of mature α factor secreted from a strain of yeast using a bioassay which relies on the inhibition of growth of a pheromone-sensitive strain of yeast. The sensitive strain of yeast used for all of the α factor bioassays in this thesis was yeast strain RC631 which is an *sst2* mutant (super sensitive to α

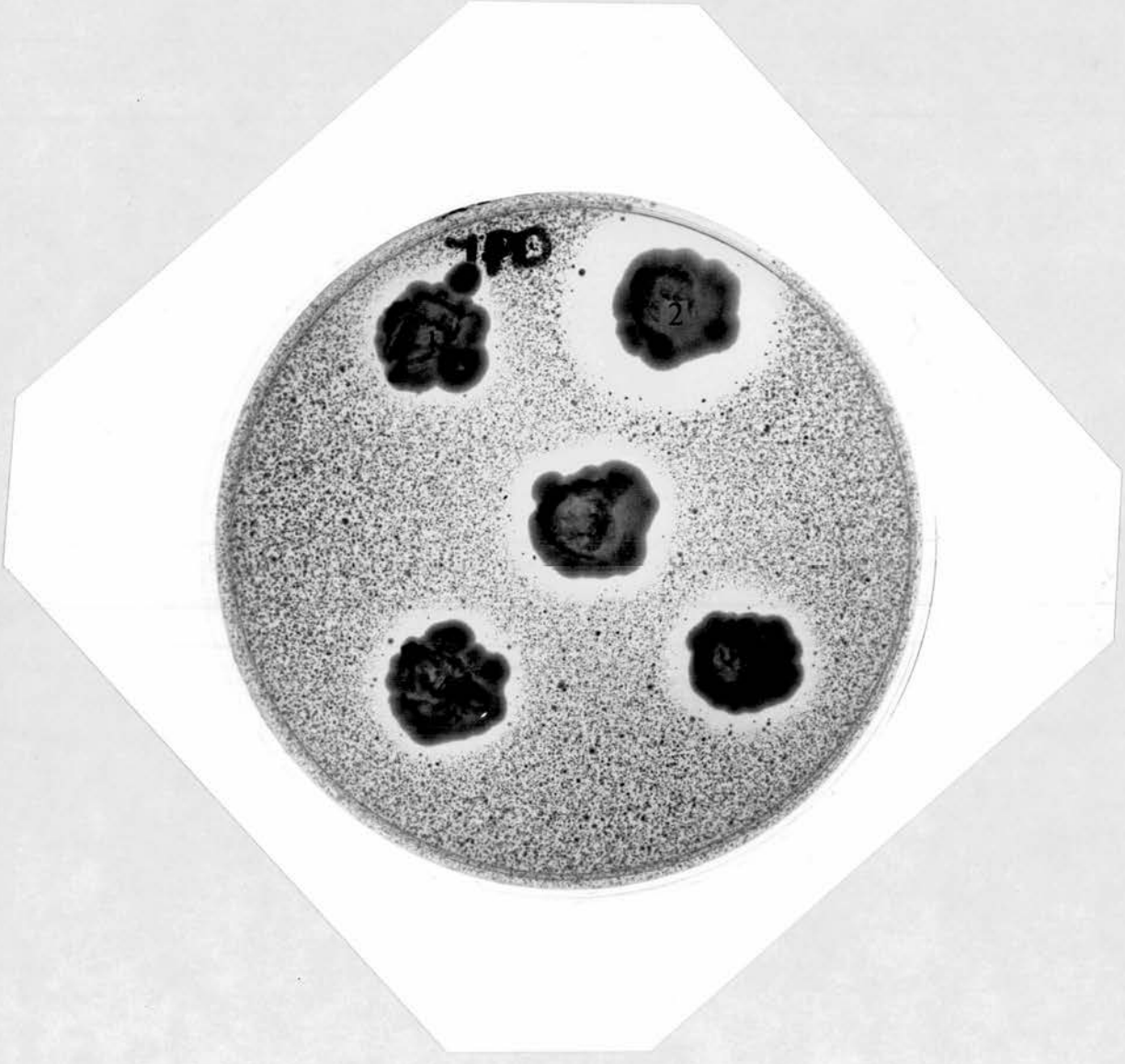
factor; Chan and Otte 1982). It was decided initially to look at the levels of secretion of mature α factor from strains of yeast expressing the different Kex2' constructs from centromere plasmids as these plasmids are present and in single copies per cell. Thus the level of expression should be the same as the level of expression of the full length Kex2p expressed from the chromosomal *KEX2* gene in wild type cells. A halo assay (see Materials and Methods) was initially used to detect the presence of α factor secreted from different strains of yeast (fig. 5.5).

The results of the halo assay show that the halo of inhibition of growth of RC631 is largest around JRY188 which suggests that it secretes the most active α factor. This was expected as JRY188 expresses the full length Kex2p which should process α factor efficiently. The halo of inhibition of growth of RC631 around PWYS3 is the smallest, also as expected as this strain does not express a functional Kex2p. The reason for the slight halo around PWYS3 will be discussed later in the chapter. The halos around the strains expressing the different Kex2' polypeptides from the centromere plasmids are all larger than the halo around PWYS3 but smaller than the halo around JRY188. This suggests that these strains do process pre-pro α factor and mature α factor is secreted but the processing is not as efficient as by the wild type Kex2p. The halo around PWYS3 (YCpKH) is slightly larger than around PWYS3 (YCpKK) and PWYS3 (YCpKS). This could possibly be because the HDEL C-terminus on the Kex2'p expressed in this strain allows retention of more Kex2'-HDEL in the cell and thus there is more α factor processed due to a greater amount of intracellular processing enzyme. We know from the *in vitro* Kex2 assays that PWYS3 (YCpKK) and PWYS3 (YCpKS) secrete Kex2 polypeptides. Thus some of the processing enzyme in these strains (that which is secreted) may not contribute to the processing of α factor or may only have a reduced contribution during the time it is in transit through the secretory pathway. The differences in the sizes of the halos around the three strains transformed with the centromere plasmids, however is only slight and may not be due to the influence of the different C-termini on the Kex2' constructs. These halo assays are essentially qualitative and a more sensitive assay that can be readily quantified was required to look at the effects that the different C-termini on the Kex2' polypeptides had on the processing of α factor.

Fig. 5.5 Halo assay for secreted α factor

The figure shows the inhibition of growth of RC631 cells (sensitive to α factor) by different strains patched onto the lawn. The halo assay was performed as described in Materials and Methods. The patched stains are as follows;-

1. PWYS3.
2. JRY188.
3. PWYS3 (YCpKH).
4. PWYS3 (YCpKK).
5. PWYS3 (YCpKS).


TPO
2

5.5 Microtitre assays for secreted α factor

In order to look at the effects of the different Kex2' polypeptides on the processing of α factor it was decided to use an assay similar to that described for the *Kluyveromyces lactis* killer toxin. This assay still relied on the inhibition of growth of RC631, but the inhibition of growth was to be measured by the density that the sensitive cells grew to in media containing α factor (or not, as the case may be) in microtitre assay plates. The greater the concentration of α factor in the media the lower the density to which the sensitive cells would grow.

The strains of yeast to be assayed for α factor secretion were grown in their respective selective media at 26°C until they reached stationary phase of growth. Cells from each of the cultures were pelleted by centrifugation at low speed, washed in YPD, pelleted again and resuspended in fresh YPD to a density of approximately 10^7 cells per ml. The cells were grown in YPD for 4 hrs and a sample of cells was taken every hour from the cultures. The cells from the samples taken each hour were immediately removed by centrifugation at high speed in a microfuge for 5 mins. The supernatants were removed, taking care not to disturb the cell pellet and transferred to a clean eppendorf tube. The supernatants from each of the strains taken at the different times were used in the assay for α factor (see Materials and Methods).

The results of the α factor assays are shown in Table 5.2. The results are expressed as growth percentage relative to an untreated control (ie YPD). Thus a result of 100% indicates no inhibition of growth of RC631 due to no detectable α factor in the media. Lower percentages indicate larger inhibitions of growth due to greater concentrations of α factor being present in the supernatants assayed.

The results show that JRY188 secretes the most α factor, as it inhibits the growth of RC631 to the greatest extent. After only 1 hr enough α factor has been secreted to inhibit the growth of RC631 to 20% of that of the control value. Supernatants from strain PWYS3 showed the lowest inhibition effect, as expected as this strain lacks a functional Kex2p, although there does nevertheless appear to be a slight inhibition of growth. After 4 hrs strain PWYS3 (YCpKH) appears to have secreted a greater

Table 5.2 Assay of alpha factor in culture supernatants of strains of yeast transformed with the centromere and episomal plasmids for the expression of different Kex2' polypeptides.

Yeast strain	Time after inoculation into fresh media (at which sample was taken)			
	1 hr	2 hrs	3 hrs	4 hrs
JRY 188	20% (3.5%)	38.4% (6.7%)	32.6% (4.4%)	15.2% (3.5%)
PWYS3	78.4% (7.4%)	85.6% (7.4%)	96.6% (2.9%)	90.9% (5.7%)
PWYS3 (YCpKH)	76.2% (3.3%)	70.8% (1.3%)	70.8% (4.6%)	61.4% (2.9%)
PWYS3 (YCpKK)	71.1% (4.6%)	74.3% (1.6%)	75.6% (2.5%)	65.1% (0.9%)
PWYS3 (YCpKS)	80.1% (1.5%)	82.5% (6.6%)	83.1% (5.7%)	71.7% (4.2%)
PWYS3 (YEpKH)	66.6% (1.9%)	64.3% (10.3%)	54.3% (8.1%)	48.9% (5.7%)
PWYS3 (YEpKK)	47.8% (3.7%)	43.0% (3.4%)	43.0% (2.5%)	26.5% (1.2%)
PWYS3 (YEpKS)	55.6% (6.6%)	55.6% (6.6%)	47.5% (2.9%)	23.3% (2.5%)

The growth of the sensitive RC631 cells in each of the alpha factor assays of cell supernatants is represented as a percentage of the density that RC631 grows to when no alpha factor is present in the media. The results are an average of three separate assays and the standard deviations from the mean are given in brackets

amount of α factor than PWYS3 (YCpKK). Also after 4 hrs PWYS3 (YCpKK) appears to have secreted more α factor than PWYS3 (YCpKS). Thus it appears that Kex2'-HDEL is more efficient at processing pre-pro α factor than Kex2'-KDEL and Kex2'-S. This is consistent with the presence of a greater amount of intracellular Kex2' polypeptide in strains expressing Kex2'-HDEL in comparison to the other strains expressing Kex2'-KDEL and Kex2'-S, and may reflect the specific retention of Kex2'-HDEL. In general the strains bearing the episomal plasmids secrete more α factor than those bearing the centromere plasmids. This suggests that the centromere plasmid bearing strains are not processing pre-pro α factor as efficiently as JRY188 or the episomal plasmid bearing strains.

The results obtained from the strains expressing the various Kex2' polypeptides from the episomal plasmids are very different from those of the centromere plasmids. Strain PWYS3 (YEpKH) appears to secrete less α factor than strains PWYS3 (YEpKK) or PWYS3 (YEpKS). This is the opposite from what was found in strains expressing the same polypeptide from the centromere plasmids. A possible explanation for the results obtained from episomal plasmid bearing strains is that the copy number of the plasmids per cell is variable between PWYS3 (YEpKH), PWYS3(YCpKK) and PWYS3 (YEpKS). This sort of copy number difference could possibly occur if the Kex2' polypeptide expressed from one of the plasmids were deleterious to the cell. In this case the Kex2'-HDEL may be deleterious to the cell if expressed at high levels, and thus the copy number of YEpKH may drop to help overcome this effect. Because of the possible copy number effects in strains transformed with the episomal plasmids it was decided to repeat the experiment with the centromere plasmid bearing strains only. Samples in the repeated experiment were taken at 1,2,4,6 and 8 hrs. The results of the α factor assays are shown in Table 5.3. Again the results are expressed as a percentage of a control in which no α factor is present. As with the previous assays PWYS3 (YCpKH) secretes more α factor than PWYS3 (YCpKK) which in turn secretes more α factor than PWYS3 (YCpKS).

A possible problem with these assays is that the Kex2 processing of pre-pro α factor is occuring extracellularly. This would not be a problem if it were not for the final

Table 5.3 Assay of alpha factor in culture supernatants of strains of yeast transformed with the centromere plasmids for the expression of the different Kex2 polypeptides

	Time after inoculation into fresh media (at which sample was taken)				
Yeast strain	1 hr	2 hrs	4 hrs	6 hrs	8 hrs
JRY 188	20.0% (2.1%)	17.8% (1.7%)	18.4% (1.9%)	16.4% (0.8%)	14.2% (1.4%)
PWYS3	96.6% (7.2%)	100% (2.9%)	100% (4.6%)	92.4% (0.6%)	92.4% (7.7%)
PWYS3 (YCpKH)	81.5% (2.3%)	80.0% (0.2%)	79.6% (1.4%)	73.3% (2.5%)	61.0% (0.2%)
PWYS3 (YCpKK)	89.0% (0.8%)	93.1% (5.1%)	90.4% (4.0%)	81.0% (0.8%)	71.2% (0.4%)
PWYS3 (YCpKS)	92.0% (1.0%)	94.2% (1.9%)	91.3% (3.2%)	84.4% (2.3%)	75.4% (1.7%)

The growth of the sensitive RC631 cells in each of the alpha factor assays of cell supernatants is represented as a percentage of the density that RC631 grows to when no alpha factor is present in the media. The results are an average of three separate assays and the standard deviations from the mean are given in brackets.

copy of the α factor pheromone in the precursor polypeptides. Cleavage by Kex2p of all but the last copy of α factor in the precusor polypeptides results in the release of Glu/Asp-Ala-Glu-Ala-α factor-Lys-Arg (see fig. 1.2). The Lys-Arg sequence at the C-terminus renders this immature α factor polypeptide at least 100-fold less active at inhibiting the growth of an *sst2* mutant (Dmochowska *et al* 1987). However the last copy of α factor (Glu-Ala-Glu-Ala-α-factor) cleaved from the precursor polypeptide does not have Lys-Arg at the C-terminus, and is active (although not to the same extent as fully mature α factor) at inhibiting the growth of an *sst2* mutant (Julius *et al* 1983). Thus if cleavage of pre-pro α factor by Kex2p were to occur extracellularly, active Glu-Ala-Glu-Ala-α factor that inhibits the growth of the sst2 mutant to some extent would be released from the precursor. Only the last copy of α factor in the precursor would cause this problem as all the other copies of α factor cleaved extracellularly would have Lys-Arg at the C-terminus rendering them essentially inactive. For all other copies of α factor apart from the final copy, cleavage by Kex2p has to occur before the removal of the Lys-Arg residues by Kex1p in order for them to mature into active α factor. As the Kex1 cleavage is intracellular, the Kex2 cleavage must also be intracellular in order for the copies of α factor other than the final copy to be able to mature into an active form.

We wanted to determine the level of processing of the α factor precursor that was taking place intracellularly. To be sure that the α factor active in inhibiting the growth of the *sst2* mutant had been processed intracellularly and not extracellularly by secreted Kex2p we made use of a plasmid called pJK6-4 (Dmochowska *et al* 1987). Plasmid pJK6-4 is a centromere plasmid that contains an altered *MFα1* gene under the control of the GAL1 promoter. This altered MFα1 gene essentially has the last copy of the α factor mating pheromone destroyed. Therefore only the first three copies of α factor can possibly be processed to give mature α factor capable of inhibiting the growth of RC631. Thus in order to get active α factor from the precursor encoded for in pJK6-4, cleavage by Kex2p must be intracellular before the removal of the C-terminal Lys-Arg residues by Kex1p. As this altered *MFα1* is under control of the GAL1 promoter, expression of the altered α factor precursor

only occurs in yeast cells transformed with pJK6-4 when grown on galactose media. In order to ensure that expression from the chromosomal copies of *MFα1* and *MFα2* did interfere with the results not use was made of the fact that JRY188 is a *sir3* mutant (Brake *et al* 1984); thus when grown at 37°C, *MFα1* and *MFα2* are not expressed, so that JRY188 and strains derived from JRY188 transformed with pJK6-4 and grown at 37°C on galactose medium will only express the altered *MFα1* gene from the GAL1 promoter. Expression from the chromosomal α factor genes will be switched off. When the pJK6-4 transformed strains of yeast were grown on glucose media at 37°C no detectable α factor was secreted from any of the strains (data not shown).

The pJK6-4 transformed strains were grown in their appropriate selective media with galactose as the sole carbon source at 37°C. The cells were harvested, washed in YPG and resuspended in YPG to a final density of approximately 10^7 cells per ml. The cells were incubated at 37°C in this medium for 14 hrs and then harvested. The supernatants were removed and different dilutions of the supernatants (diluted with YPG) from each of the strains were used to assay for α factor using the microtitre assay. The results of the assay are shown in Table 5.4 and fig 5.6.

JRY188 (pJK6-4) appears to secrete the most active α factor as shown by the greatest inhibition of growth of RC631. This is consistent with the results of the previous α factor assays. The strain PWYS2 (pJK6-4) appears to secrete the least α factor which is also consistent with the previous α factor assays. The strains PWYS2 (YCpKH, pJK6-4) and PWYS2 (YCpKK pJK6-4) secrete similar amounts of active α factor, but more than PWYS2 (YCpKS pJK6-4). The lower amount of α factor secreted from the YCpKS harbouring strain is consistent with previous results but in previous assays the YCpKH harbouring strain has always secreted more α factor than the YCpKK harbouring strains. These results suggest that the Kex2' polypeptides with the different C-termini can all process α factor intracellularly to different extents.

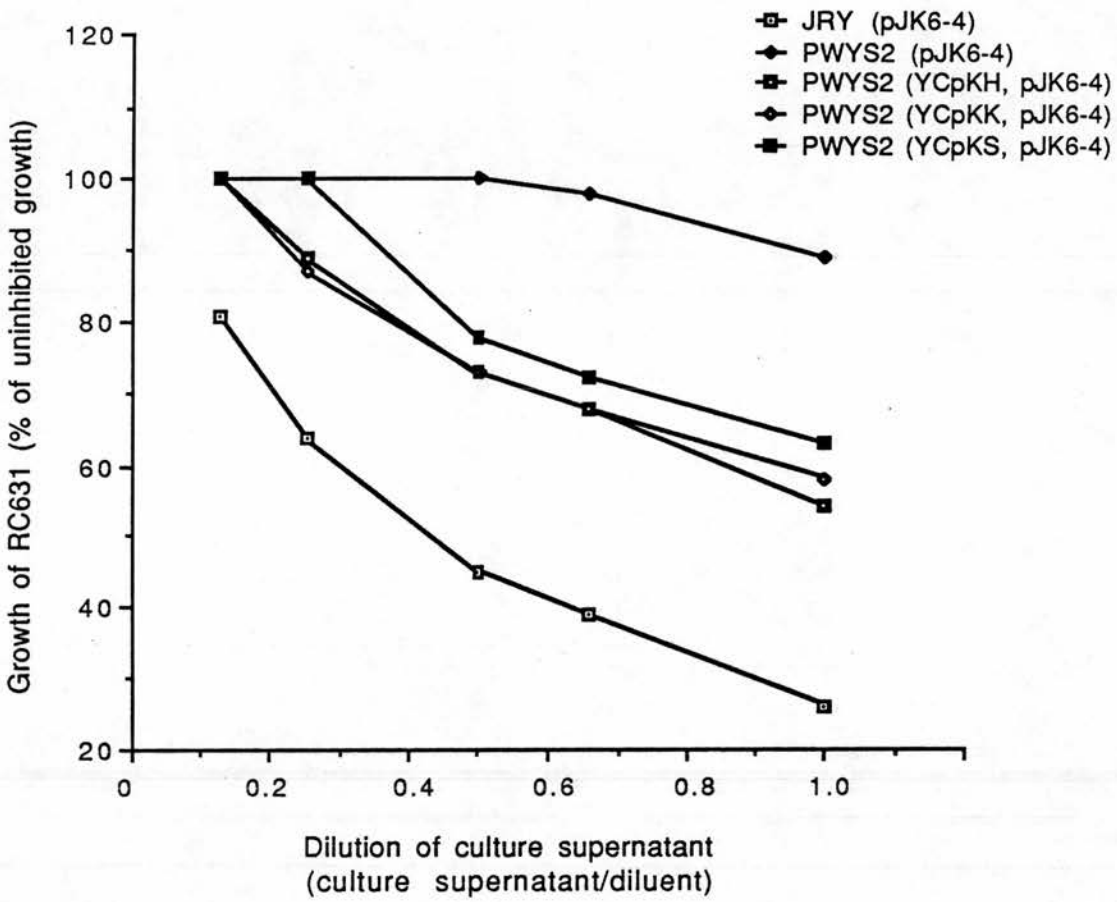
Table 5.4 Assay of alpha factor processed from pJK6-4 encoded precursor in culture supernatants of strains of yeast transformed with the centromere plasmids encoding for the different Kex2' polypeptides

Yeast strain	Dilution of culture supernatant				
	No dilution	2 / 3 dilution	1 / 2 dilution	1 / 4 dilution	1 / 8 dilution
JRY (pJK6-4)	26% (1.6%)	39% (2.2%)	45% (1.5%)	64% (0.5%)	81% (2.0%)
PWYS2 (pJK6-4)	89% (0.6%)	98% (4.3%)	100% (-)	100% (-)	100% (-)
PWYS2 (YCpKH, pJK6-4)	54% (1.6%)	68% (0.6%)	73% (1.4%)	89% (1.3%)	100% (-)
PWYS2 (YCpKK, pJK6-4)	58% (0.2%)	68% (1.4%)	73% (0.8%)	87% (1.1%)	100% (-)
PWYS2 (YCpKS, pJK6-4)	63% (1.6%)	72% (4.0%)	78% (2.8%)	100% (-)	100% (-)

The growth of the sensitive RC631 cells in each of the alpha factor assays of cell supernatants is represented as a percentage of the density that RC631 grows to when no alpha factor is present in the media. The results are an average of three seperate assays and the standard deviations from the mean are given in brackets. The supernatants all came from cells grown in YPG at 37 C for 14 hours and the supernatants were diluted with YPG.

Fig. 5.6 A graph to show the growth inhibition of RC631 exposed to culture supernatants of different strains of yeast (I).

The results of microtitre assays for α factor in the culture supernatants of different strains of yeast, processed from the pJK6-4 encoded precursor (shown in Table 5.4) are plotted. The dilution of culture supernatant (culture supernatant/diluent) from the different strains is plotted against the growth of RC631 (the α factor sensitive strain) expressed as a percentage of uninhibited growth.


JRY (pJK6-4)
PWYS2 (pJK6-4)
PWYS2 (YCpKH, pJK6-4)
PWYS2 (YCpKK, pJK6-4)
PWYS2 (YCpKS, pJK6-4)
Growth of RC631 (% of uninhibited growth)
120
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
Dilution of culture supernatant
(culture supernatant/diluent)

5.6 Concluding remarks

The results of the *in vitro* and *in vivo* assays for Kex2 activity show some interesting trends especially when the different truncated Kex2p's are expressed from the centromere plasmids. The apparently efficiently retained Kex2'-HDEL polypeptide processes pre-pro α factor to a greater extent than the secreted Kex2'-S polypeptide. The Kex2'-KDEL polypeptide although not as efficiently retained within the cell as Kex2'-HDEL does appear to be retained to a greater extent than Kex2'-S; this could possibly be due to weak recognition of the KDEL signal by the yeast retention system. It has been reported (Lewis *et al* 1990) that the *S. cerevisiae ERD2* gene product (the putative HDEL receptor) weakly recognises the KDEL sequence, therefore it is not suprising that we see some apparent retention of Kex2'-KDEL in yeast. Another observation was that in the Kex2$^-$ mutants there was still a very low level of Kex2 activity, both in the *in vitro* assays and the *in vivo* assays. This "background" activity could possibly be due to non-specific proteases or alternatively due to an aspartyl protease that reportedly allows the *KEX2* independent processing of pre-pro α factor in yeast (Egel-Mitani *et al* 1990). In either case the "background" activity is very low in the *in vitro* and *in vivo* assays and does not affect our interpretation of the effect of the different Kex2' constructs.

Chapter six.

Construction of plasmids for the expression of a Kex2-Protein disulphide isomerase fusion protein in yeast, and the *in vitro*/*in vivo* Kex2 activities of this polypeptide.

6.0 Introduction

In the previous chapter the addition of the tetrapeptide HDEL at the C-terminus of Kex2'p appears to have some effect on the efficiency of retention within the cell of Kex2' polypeptides when compared with the other C-termini. However, the HDEL signal does not result in full retention of Kex2' polypeptides as Kex2'-HDEL is secreted when expressed from an episomal plasmid. One possible explanation for this would be saturation of the retention system when Kex2'-HDEL is expressed from an episomal plasmid. Alternatively it is possible that Kex2'-HDEL is not efficiently recognised by the retention system because the HDEL signal is buried in the structure of the polypeptide. It has been reported that the yeast retention system is quite leaky and easily saturable in comparison to the mammalian retention system (Pelham *et al* 1988, Dean and Pelham 1990). It has also been reported that the C-terminal HDEL signal may not be recognised by its putative receptor in at least some protein constructs due to masking of the C-terminus (Pelham *et al* 1988). If the retention system is easily saturable it is difficult to explain how abundant HDEL-containing proteins such as BiP and PDI are not normally secreted from the cell if the HDEL recognition system is the only method of retention. It is important to note that the levels of expression of the Kex2' polypeptides with the different C-termini are expected to be low, even in cells transformed with the episomal plasmids, since the Kex2 promoter is a weak promoter; we would not therefore expect to saturate a retention system that can reportedly retain abundant proteins such as BiP and PDI in the cell with the comparatively low levels of Kex2'-HDEL expected to be expressed in PWYS3 (YEpKH). It therefore seems most likely that the secretion of Kex2'-HDEL from PWYS3 (YEpKH) results not from saturation of the retention system but rather from inefficient recognition of the HDEL signal by its putative receptor because of masking.

6.1 Attachment of the PDI C-terminus to a Kex2' polypeptide.

Because of this possibility that the HDEL signal on Kex2'-HDEL was not being recognised efficiently due to masking, I decided to attach a C-terminal domain derived from a protein known to be efficiently retained in the ER to Kex2'. Specifically I decided to attach the C-terminus of PDI to make a Kex2'-PDI fusion protein with the HDEL sequence of PDI at the extreme C-terminus of the hybrid protein. The yeast

PDI gene and sequence were made available by Dr. M. Tuite and Dr. R. Freedman. The PDI gene was supplied in a plasmid named pRF16. It was decided, because of convenient restriction sites to attach a DNA fragment coding for the 60 C-terminal amino acids of yeast PDI (fig. 6.1) to the truncated KEX2 gene in both centromere and episomal plasmids (fig. 6.2). The resulting plasmids named YCpKP and YEpKP respectively both encoded the same Kex2'-PDI fusion protein. The plasmids were transformed into the strains PWYS2 and PWYS3.

6.2 Assays for Kex2 activity *in vitro*

The assays for Kex 2 activity were performed as in the previous chapter with the additional strains of yeast PWYS3 (YCpKP) and PWYS3 (YEpKP) included. The results of the assays are shown in Table 6.1 and fig. 6.3. These results show that as before there is essentially no detectable Kex2 activity in PWYS3. The overall Kex2 activities in the strains bearing the episomal plasmids was again higher than those bearing the centromere plasmids as expected. It is interesting to note that strain PWYS3 (YCpKP) does not secrete any detectable Kex2'-PDI into the supernatant as is the case with PWYS3 (YCpKH) not secreting any detectable Kex2'-HDEL into the supernatant. There was, however detectable intracellular Kex2 activity in PWYS3 (YCpKP) suggesting that the Kex2'-PDI hybrid protein is active. These results suggest that the Kex2'-PDI hybrid protein is retained within the cell when expressed from a centromere plasmid. There was detectable Kex2 activity in the supernatants of PWYS3 (YCpKK) and PWYS3 (YCpKS) as had been observed previously which suggests that the KDEL and stop sequences at the C-terminus of the truncated Kex2p do not have as great an effect as the HDEL and PDI sequences at retaining Kex2' polypeptides in the cell.

The internal and external Kex2 activities of the strains transformed with the episomal plasmids also show some interesting features. Strain PWYS3 (YEpKP) has a much higher internal Kex2 activity than any of the other strains, but also the lowest external Kex2 activity. Thus it seems that the addition of the 60 C-terminal amino acids of PDI to the C-terminus of the Kex2' polypeptide increases the ability of this protein to be retained within the cell. This could be because the addition of these extra amino acids allows the HDEL sequence to be presented in such a way that it is more efficiently recognised by the putative HDEL receptor than when these

Fig. 6.1 The DNA fragment encoding the 60 C-terminal amino acids of yeast protein disulphide isomerase.

The diagram shows the Sau 3A fragment from plasmid pRF16 (obtained from Dr. M. Tuite and Dr. R. Freedman) and the 60 C-terminal of yeast PDI that it encodes. This fragment of DNA was used to create a gene fusion with the DNA encoding Kex2' that codes for a Kex2'-PDI hybrid polypeptide (fig. 6.2).

Sau3A

AGA TCC TTG GAC TCT TTA TTC GAC TTC ATC AAG

R S L D S L F D F I K

Sal1

GAA AAC GGT CAC TTC GAC GTC GAC GGT AAG GCC

E N G H F D V D G K A

TTG TAC GAA GAA GCC CAG GAA AAA GCT GCT GAG

L Y E E A Q E K A A E

GAA GCC GAA GCT GAC GCC GAA GCC GAA GCT GAC

E A E A D A E A E A D

GCT GAC GCT GAA TTG GCT GAC GAA GAA GAT GCC

A D A E L A D E E D A

Sau3A

ATT CAC GAT GAA TTG TAA TTC CTG ATC

I H D E L *

Fig. 6.2 Construction of vectors for the expression of Kex2'-PDI in yeast

Plasmids YcpKH and YEpKH were digested with Bam HI. The purified Sau 3A fragment from plasmid pRF16 (see fig. 6.1) was ligated with the Bam HI digested YCpKH or YEpKH and then transformed into *E. coli* strain NM522. Recombinant plasmids were screened for by the introduction of a Sal I site into the vectors. Recombinant plasmids then had to be checked to deduce in which orientation the Sau 3A fragment had been cloned. The orientation was determined making use of the assymetric Sal I site and also the fact that one of the Sau 3A sites when cloned into the Bam HI site re-created a Bam HI site. In the correct orientation the PDI open reading frame is in frame with the Kex2' open reading frame and thus a Kex2'-PDI hybrid protein is encoded for. The resulting plasmids for the expression of Kex2'-PDI were called YCpKP and YEpKP (single copy and multi-copy in yeast respectively).

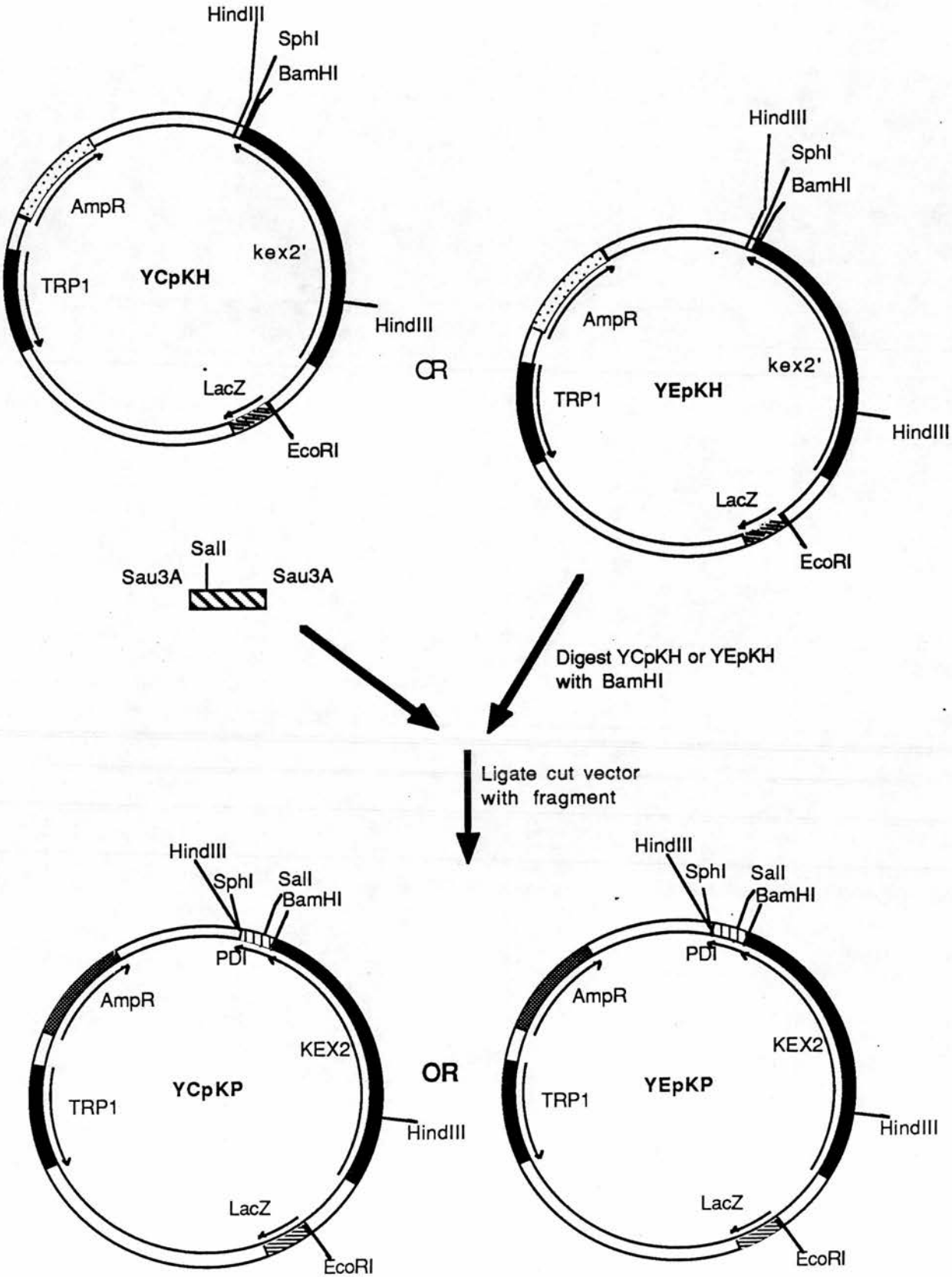

HindIII
SphI
BamHI
AmpR
kex2'
TRP1
YCpKH
HindIII
LacZ
EcoRI
OR
HindIII
SphI
BamHI
AmpR
kex2'
TRP1
YEpKH
HindIII
LacZ
EcoRI
SalI
Sau3A
Sau3A
Digest YCpKH or YEpKH
with BamHI
Ligate cut vector
with fragment
HindIII
SphI
SalI
BamHI
PDI
AmpR
KEX2
TRP1
YCpKP
HindIII
LacZ
EcoRI
OR
HindIII
SphI
SalI
BamHI
PDI
AmpR
KEX2
TRP1
YEpKP
HindIII
LacZ
EcoRI

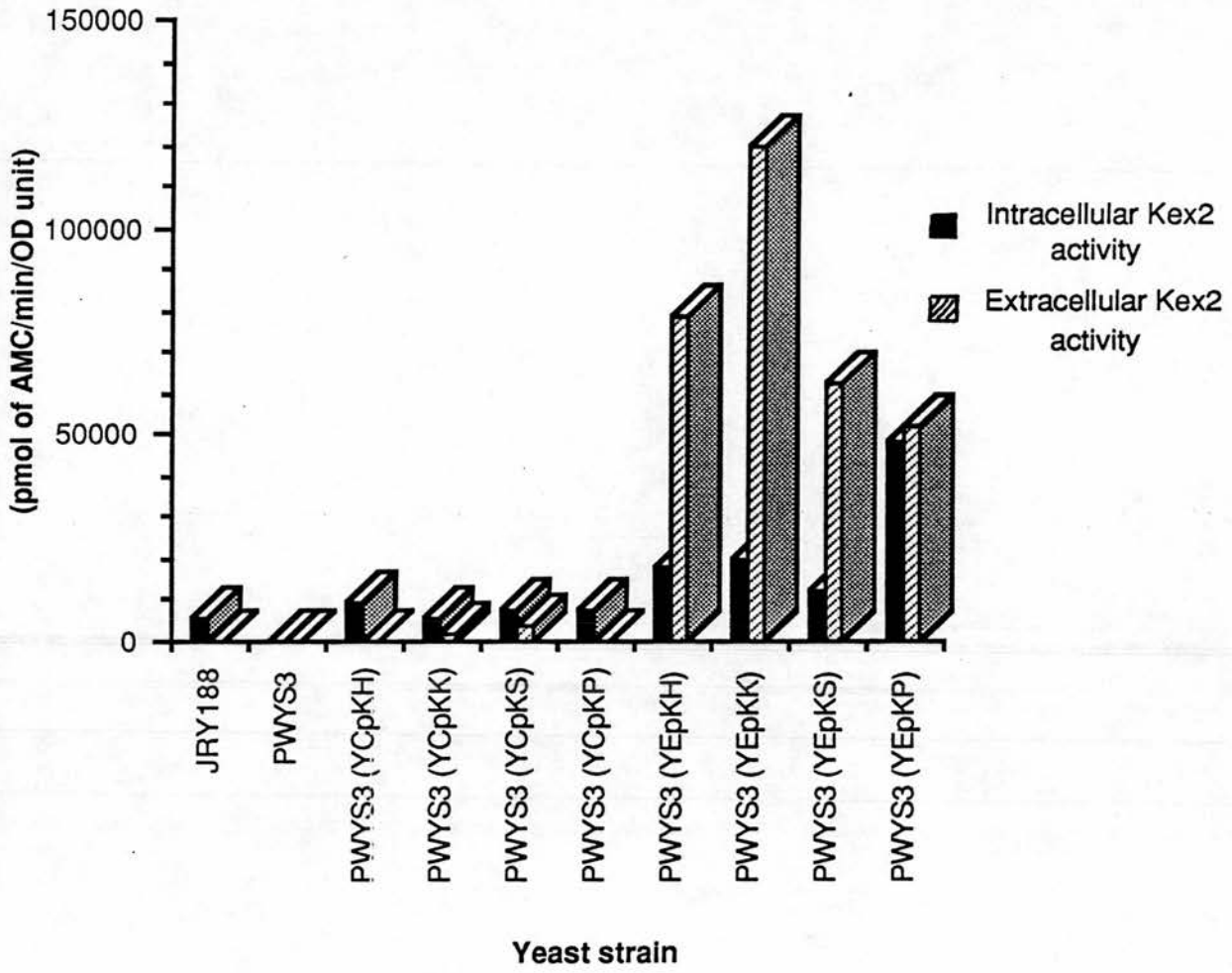
Table 6.1 The intracellular and extracellular Kex2 activities of yeast strains expressing the different Kex2' polypeptides.

Yeast strain	Internal Kex2 activity	external Kex2 activity
JRY 188	5.5×10^3 (4.1×10^2)	0 (-)
PWYS3	8.8×10^1 (3.5×10^1)	0 (-)
PWYS3 (YCpKH)	9.3×10^3 (1.7×10^3)	0 (-)
PWYS3 (YCpKK)	5.4×10^3 (1.2×10^3)	1.5×10^3 (1.0×10^2)
PWYS3 (YCpKS)	7.9×10^3 (1.1×10^3)	3.9×10^3 (1.8×10^2)
PWYS3 (YCpKP)	7.8×10^3 (6.7×10^2)	0 (-)
PWYS3 (YEpKH)	1.8×10^4 (1.1×10^3)	7.9×10^4 (9.5×10^3)
PWYS3 (YEpKK)	2.0×10^4 (1.2×10^3)	1.2×10^5 (1.7×10^4)
PWYS3 (YEpKS)	1.2×10^4 (9.6×10^2)	6.3×10^4 (8.0×10^3)
PWYS3 (YEpKP)	4.8×10^4 (1.6×10^3)	5.2×10^4 (1.0×10^4)

The Kex2 activities are in picomoles of AMC released from bQRR-MCA per minute, per OD unit of cells. The results are an average of three separate Kex2 assays carried out in standard conditions (see materials and methods), and the standard deviations from the mean are shown in brackets. Intracellular Kex2 activities were obtained by assaying Brij permeabilised cells(see materials and methods) of cells from each strain. Extracellular activities were obtained by assaying the supernatants of each strain grown in selective media.

Fig 6.3 A graph showing the intracellular and extracellular Kex2 activities of yeast strains expessing the different Kex2' polyeptides (II).

The *in vitro* intracellular and extracellular Kex2 activities (shown in Table 6.1) for the strains expressing the different Kex2' polypeptides are plotted. The Kex2 activities are in pico-moles of AMC released from the synthetic peptide bQRR-MCA per minute, per OD unit of cells (pmol of AMC/min/OD unit).


150000
100000
50000
0
(pmol of AMC/min/OD unit)
Intracellular Kex2 activity
Extracellular Kex2 activity
JRY188
PWYS3
PWYS3 (YCpKH)
PWYS3 (YCpKK)
PWYS3 (YCpKS)
PWYS3 (YCpKP)
PWYS3 (YEpKH)
PWYS3 (YEpKK)
PWYS3 (YEpKS)
PWYS3 (YEpKP)
Yeast strain

extra amino acids are not present. It is also possible that there is something additional in the 60 C-terminal amino acids of PDI that increases the efficiency of retension of the Kex2'-PDI hybrid protein over the other constructs. Although the addition of the 60 C-terminal amino acids of PDI to the Kex2' polypeptide appears to greatly increase the efficiency of retention, there is still Kex2 activity in the supernatants of strains expressing this polypeptide from episomal plasmids. It therefore appears that even for this Kex2'-PDI construct the retention system is not 100% efficient.

6.3 Assays for α factor.

The α factor assays were performed as in the previous chapter on the pJK6-4 transformed strains with the additional strain PWYS2 (YCpKP, pJK6-4) also being assayed. The results of the microtitre assays for α factor are shown in Table 6.2. The results of the α factor assays show that the strain PWYS2 (pJK6-4) secretes the least α factor of the strains tested, as expected. The strains PWYS2 (YCpKH/YCpKK/YCpKS, pJK6-4) all show similar trends in α factor processing as shown in the previous chapter. Suprisingly however, results documented in Table 6.2 show that PWYS2 (YCpKP, pJK6-4) appears to be the *least* efficient at processing pre-pro α factor of the strains expressing the Kex2' polypeptides from centromere plasmids. Therefore although the Kex2'-PDI hybrid protein is apparently more efficiently retained within the cell than the other Kex2' constructs it is not able to mature α factor as efficiently as the other constructs. There are a number of possible explanations for this. Firstly it is possible that the Kex2'-PDI hybrid protein is able to cleave the synthetic peptide bQRR-MCA in the *in vitro* assay but not process the α factor precursor efficiently *in vivo* ; if this hybrid protein is efficiently localised to the ER it may not be able to process the α factor precursor because of the intraorganellar environment in the ER (processing by Kex2p in wild type cells occurs in the Golgi). A second possibility is that if α factor precursor is processed in the ER, the α factor peptide cannot be matured into its active secreted form. In other words premature processing by Kex2 may effect subsequent processing events and/or secretion of α factor.

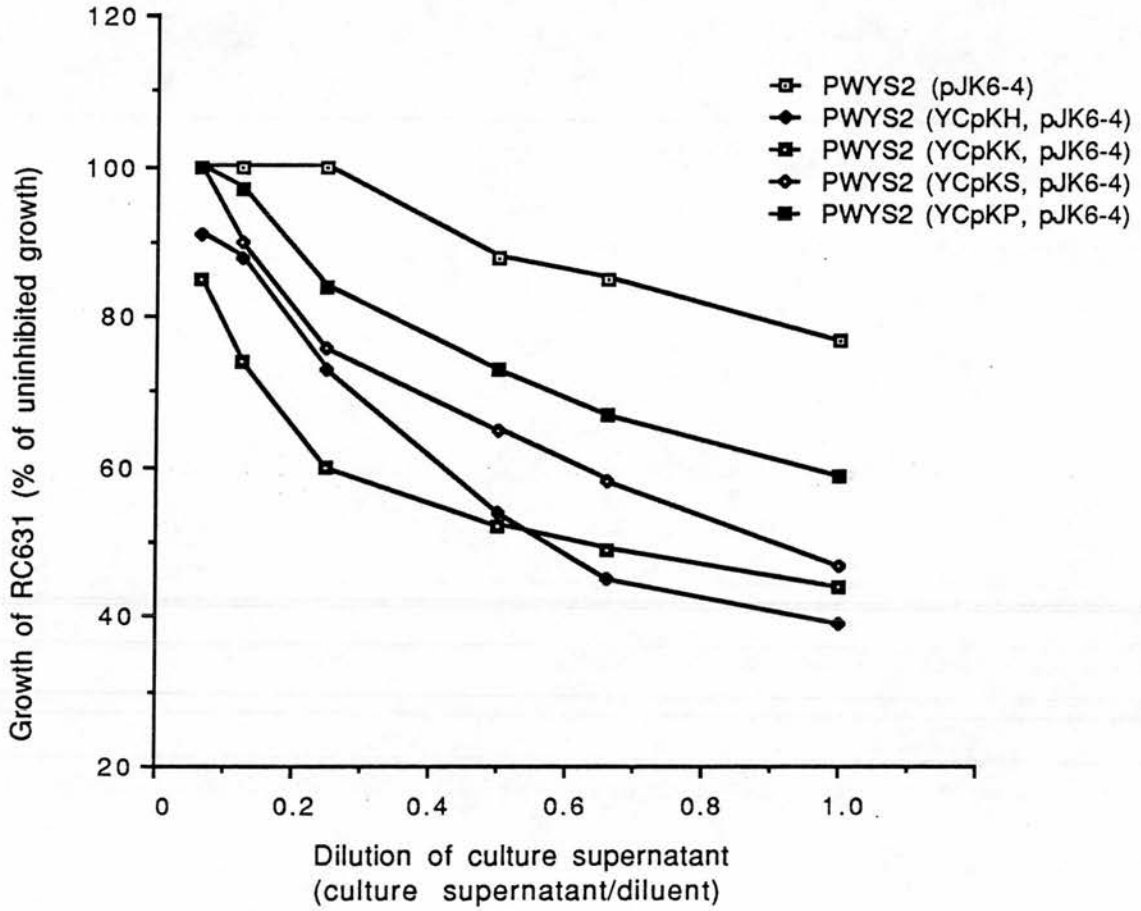
Table 6.2 Assay of alpha factor processed from pJK6-4 encoded precursor in culture supernatants of strains of yeast transformed with the centromere plasmids for the expression of the different Kex2' polypeptides.

Yeast strain	Dilution of culture supernatant					
	No dilution	2 / 3 dilution	1 / 2 dilution	1 / 4 dilution	1 / 8 dilution	1 / 1 6 dilution
PWYS2 (pJK6-4)	77% (9.2%)	85% (1.7%)	88% (1.4%)	100% (-)	100% (-)	100% (-)
PWYS2 (YCpKH, pJK6-4)	39% (6.6%)	45% (1.2%)	54% (1.5%)	73% (2.7%)	88% (3.6%)	91% (1.6%)
PWYS2 (YCpKK, pJK6-4)	44% (4.8%)	49% (5.2%)	52% (2.4%)	60% (2.1%)	74% (0.7%)	85% (3.0%)
PWYS2 (YCpKS, pJK6-4)	47% (0.4%)	58% (4.3%)	65% (4.8%)	76% (0.7%)	90% (3.4%)	100% (-)
PWYS2 (YCpKP, pJK6-4)	59% (0.2%)	67% (1.9%)	73% (1.6%)	84% (1.9%)	97% (9.6%)	100% (-)

The growth of the sensitive RC631 cells in each of the alpha factor assays of cell supernatants is represented as a percentage of the density that RC631 grows to when no alpha factor is present in the media. The results are an average of three seperate assays and the standard deviations from the mean are given in brackets. The supernatants all came from cells grown in YPG at 37 C for 14 hours and the supernatants were diluted with YPG.

Fig. 6.4 A graph to show the growth inhibition of RC631 exposed to culture supernatants of different strains of yeast (II).

The results of microtitre assays for α factor in the culture supernatants of different strains of yeast, processed from the pJK6-4 encoded precursor (shown in Table 6.2) are plotted. The dilution of culture supernatant (culture supernatant/diluent) from the different strains is plotted against the growth of RC631 (the α factor sensitive strain) expressed as a percentage of uninhibited growth.


120
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
Growth of RC631 (% of uninhibited growth)
Dilution of culture supernatant
(culture supernatant/diluent)
PWYS2 (pJK6-4)
PWYS2 (YCpKH, pJK6-4)
PWYS2 (YCpKK, pJK6-4)
PWYS2 (YCpKS, pJK6-4)
PWYS2 (YCpKP, pJK6-4)

6.4 Concluding remarks.

Attachment of the 60 C-terminal amino acids of PDI to the Kex2' polypeptide greatly increases the efficiency of retention of Kex2 activity within the cell. The increased retention of the Kex2' polypepeptide due to the PDI C-terminus being attached to it results in a decrease in secretion of mature α factor.

Chapter seven.

The production of polyclonal antibodies to specifically recognise Kex2 endopeptidase.

7.0 Introduction.

In order to further study the location and efficiency of retention of the various Kex2' constructs with the different C-termini it was decided to use immunological techniques. These techniques require antibodies that will specifically recognise Kex2p in the presence of other yeast proteins. The first step in raising specific antibodies is the preparation of an antigen that contains Kex2p epitopes. Kex2p is a membrane protein and is present in low abundance in *S.cerevisiae*, therefore it is not feasible to purify the wild type protein in sufficient abundance to use it as an antigen. However, as the *KEX2* gene was available and the sequence known it was decided to use recombinant DNA technology to produce a specific Kex2 antigen that could be used to immunise rabbits.

Using recombinant DNA technology it is possible to construct hybrid in-frame gene fusions that can be expressed in *E.coli* at high levels to produce hybrid fusion proteins that can be purified and used as immunogens. It was decided to make a hybrid *Staphylococcal aureus* protein A-Kex2 fusion protein to raise antibodies, as protein A fusion proteins offer a number of practical advantages (Lowenadler *et al* 1986):- Fusion with the protein A moiety allows affinity purification of the fusion protein with IgG linked to a solid matrix with a good yield (Nilsson *et al* 1985). The protein A moiety probably enhances the immune response due to its repetitive structure (Lowenadler *et al* 1986). Also, the recombinant protein produced in *E.coli* can easily be identified and checked with respect to predicted size by Western blotting because of the ability of the protein A portion to bind IgGs.

In this chapter I will describe the construction and purification of a protein A-Kex2 fusion protein and its use as an immunogen. Also the construction and use of a β-galactosidase-Kex2 fusion protein in affinity purification of specific anti-Kex2 antibodies from crude rabbit immune serum will be described.

7.1 The Protein A fusion vector

There are several protein A fusion vectors commercially available for the production of fusion proteins in *E.coli*, with different multiple cloning sites allowing fusions of different open reading frames with protein A. However for the purpose of producing a protein A-Kex2 fusion protein a plasmid called pKPRA was used (constructed by Alan Boyd). The plasmid pKPRA (see fig. 7.2) carries:- a kanamycin resistance gene and a segment of DNA encoding two and a half repeats of the five IgG binding domains of protein A (fig. 7.1), placed in-frame with the ATG of lacZ to put it under control of the lac promoter. Also the 3' end of the protein A portion is in frame with the lacZ gene thus creating a protein A-lacZ fusion. When suitable strains of *E.coli* (eg NM522) are transformed with pKPRA and plated onto X-Gal plates, blue colonies are produced. Cloning of an in frame open reading frame (ORF) into the 3' end of the protein A segment of DNA usually disrupts the open reading frame of the protein A-lacZ fusion (a protein A-ORF fusion protein is produced instead) and therefore white colonies of *E.coli* are produced on X-Gal plates. This vector can be used for making protein A fusion proteins in large amounts and has the advantage of a colour test on X-Gal plates to aid in screening for the correct recombinant constructs. The construction of pKPRA is shown in (fig. 7.2).

7.2 The fusion of Kex2 to the 3' end of protein A in pKPRA.

The protein A-Kex2 hybrid protein was created by cloning a portion from the N-terminal coding region of the *kex2'* gene from YCpKS into pKPRA. The portion of the gene used extended from the Hind III site in *kex2'* to the Hind III site in the remainder of the polylinker in YCpKS (see fig. 7.3). The Hind III fragment was made blunt ended by filling in the Hind III ends. This filled-in fragment was cloned into the filled-in Bam HI site of pKPRA to create pKPRA Kex. As the fragment could be cloned in either orientation, and the orientation of the fragment is important, the constructs were checked for the correct orientation with suitable restriction digests. The Hind III filled-in fragment cloned into the BamHI filled in site of pKPRA in the correct orientation creates a hybrid gene that encodes a 75K predicted molecular weight protein A-Kex2 fusion protein. Analysis of pKPRA Kex by restriction analysis revealed (as far as it is possible to tell) that the correct construct had been made, but it still remained to be seen whether a fusion protein of the predicted size

Fig 7.1 A schematic map of pRIT11, showing the protein A gene and the domain structure of protein A.

pRIT11 contains the protein A gene from *Staphylococcus aureus* with a polylinker at the C-terminal coding region of the gene. The domains of the protein shown are: S a signal sequence, A-D which are IgG domains, E which is a region homologous to A-D and X which is the C-terminal part of protein A that lacks IgG binding activity. In the construction of pKPRA (fig. 7.2) the Hind III/Bam HI fragment of pRIT11 coding for two and a half IgG binding domains and the C-terminal region of protein A was used.

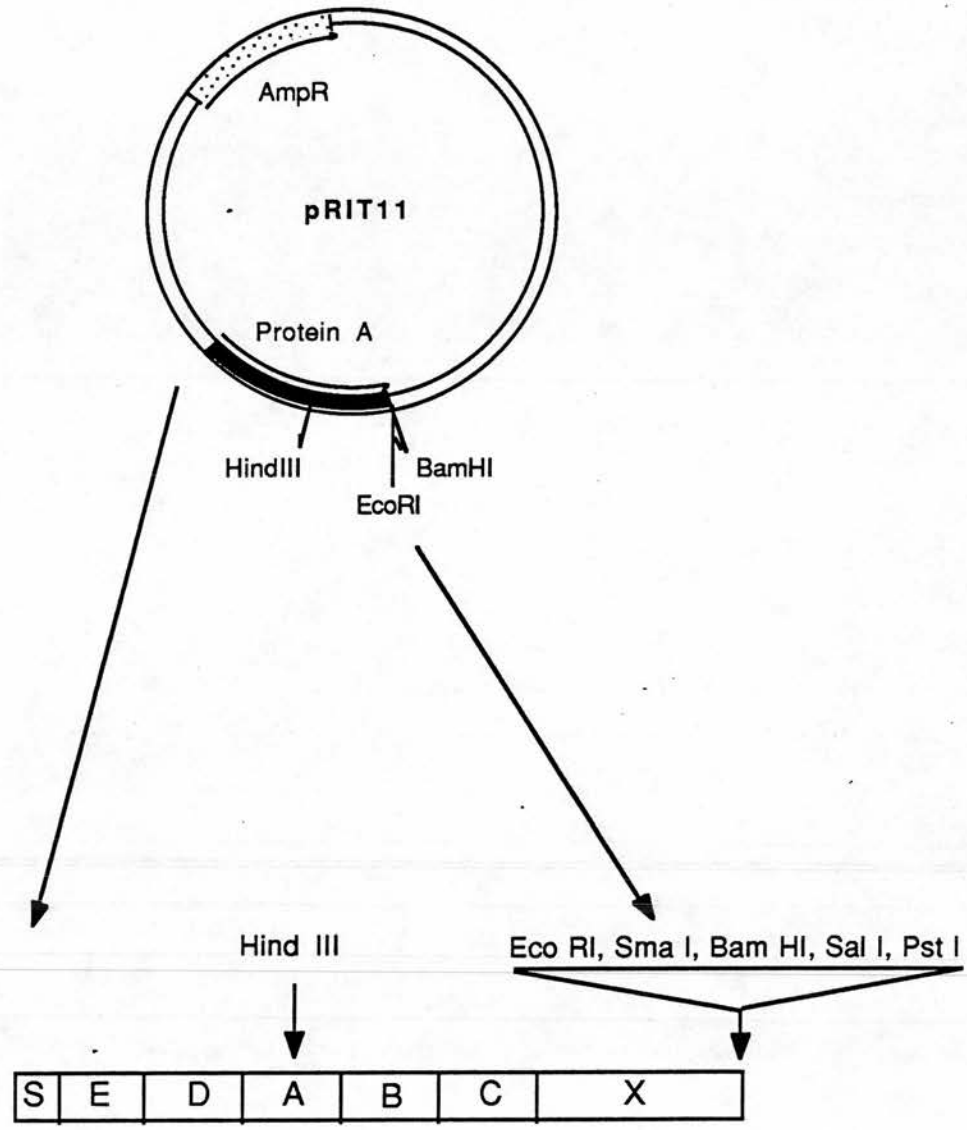

AmpR
pRIT11
Protein A
HindIII
BamHI
EcoRI
Hind III
Eco RI, Sma I, Bam HI, Sal I, Pst I
S
E
D
A
B
C
X

Fig. 7.2 Construction of plasmid pKPRA

This plasmid was constructed by Dr Alan Boyd and is presented in this thesis for completeness. For this construction plasmid pRIT11 (Lowenadler *et al* 1986) was used as a source of protein A DNA. The pRIT11 DNA was digested with Hind III and Bam HI which releases a fragment of DNA coding for two and a half repeats of the five IgG binding repeats of protein A. This Hind III/Bam HI fragment was ligated with pK19E DNA which had been Hind III/Bam HI digested. Plasmid pK19E was made by Eco RI digesting pK19-filling in the EcoRI ends to make them blunt ended, and the re-ligating the vector. The reason for cloning into pK19E and not pK19 was so that the LacZ gene is in frame with the protein A gene portion being cloned in. Plasmid pKPRA encodes a protein A-LacZ fusion protein of approximately 26K and E.coli strain NM522 when transformed with this plasmid gives blue colonies on X-Gal plates.

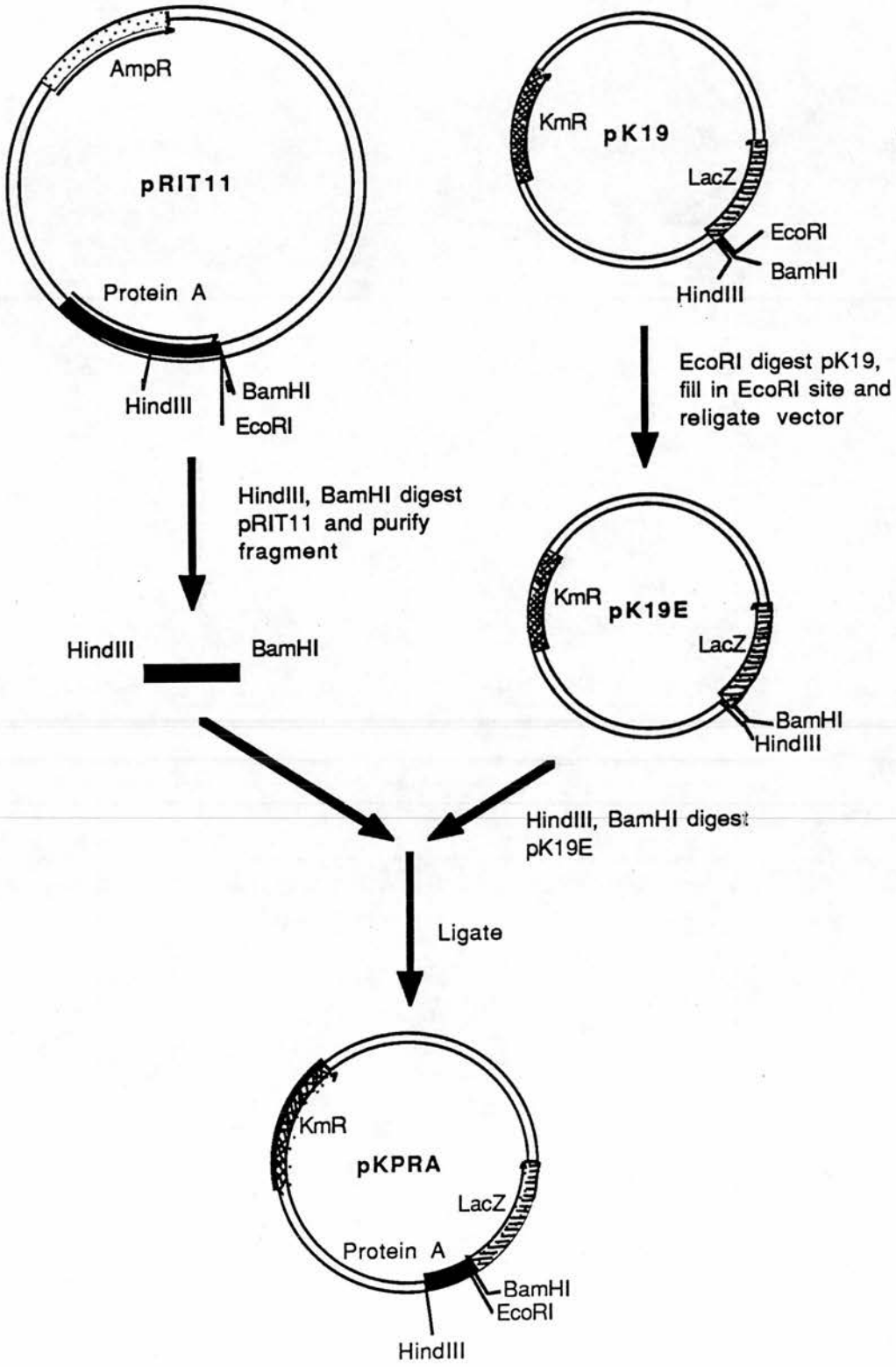
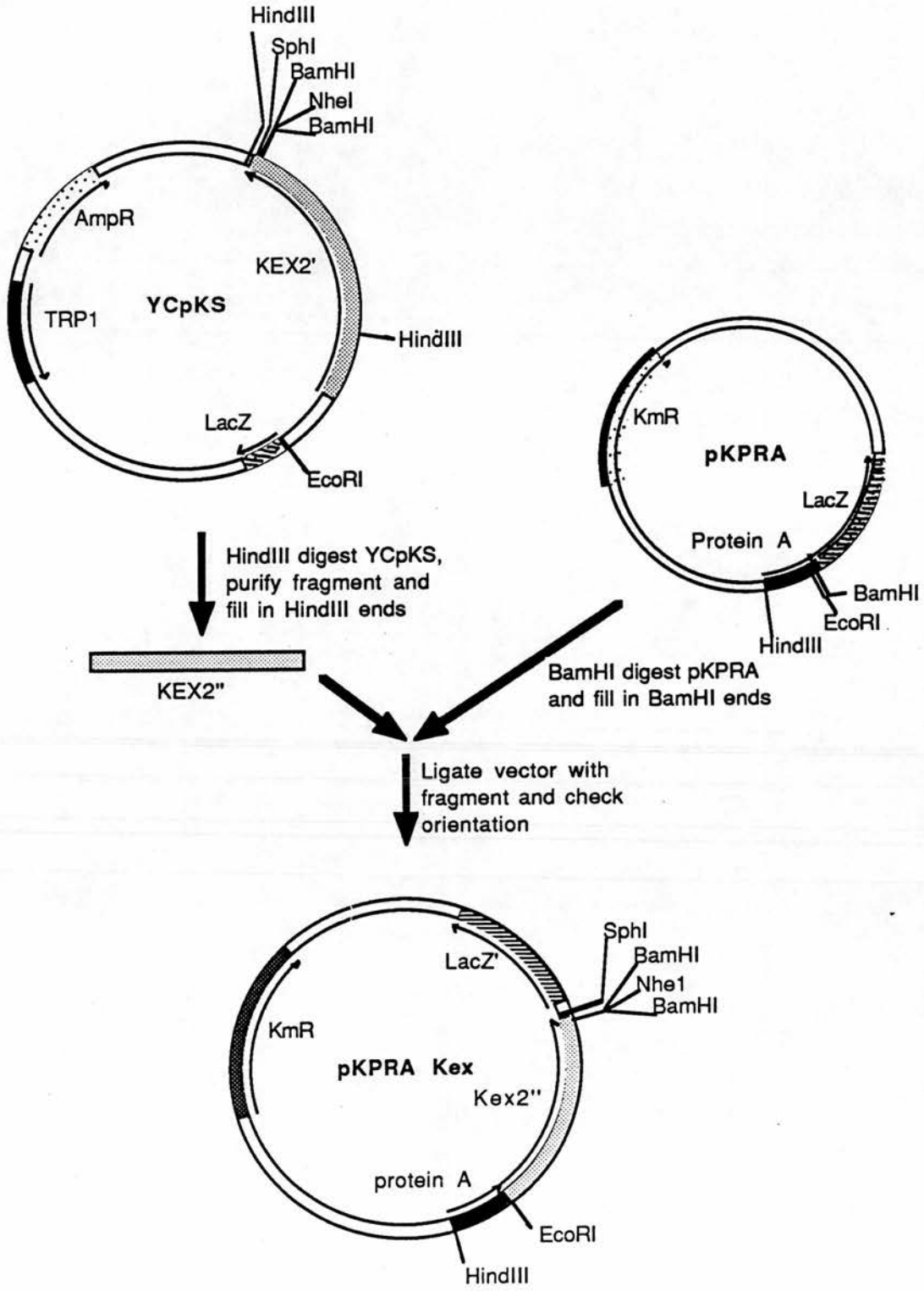

AmpR
pRIT11
Protein A
HindIII
BamHI
EcoRI
HindIII, BamHI digest
pRIT11 and purify
fragment
HindIII
BamHI
KmR
pK19
LacZ
EcoRI
BamHI
HindIII
EcoRI digest pK19,
fill in EcoRI site and
religate vector
KmR
pK19E
LacZ
BamHI
HindIII
HindIII, BamHI digest
pK19E
Ligate
KmR
pKPRA
LacZ
Protein A
BamHI
EcoRI
HindIII

Fig. 7.3 Construction of the protein A-KEX2 fusion plasmid pKPRA-Kex

For this construction, plasmid YCpKS was used as a source of KEX2 DNA. YCpKS was digested with Hind III and the ends made blunt by filling in. Plasmid pKPRA was digested with Bam HI and the ends made blunt by filling in. The DNA's were ligated together, transformed into NM522 and transformants harbouring a plasmid with the Kex2 insert in the correct orientation were selected. The resulting plasmid pKPRA-Kex encodes a protein A-Kex2 gene fusion in which the protein A and Kex2 coding sequences are in frame. The predicted size of the fusion protein is approximately 75K.


HindIII
SphI
BamHI
NheI
BamHI
AmpR
KEX2'
YCpKS
TRP1
HindIII
LacZ
EcoRI
KmR
pKPRA
LacZ
Protein A
BamHI
EcoRI
HindIII
HindIII digest YCpKS, purify fragment and fill in HindIII ends
KEX2"
BamHI digest pKPRA and fill in BamHI ends
Ligate vector with fragment and check orientation
LacZ'
SphI
BamHI
Nhe1
BamHI
KmR
pKPRA Kex
Kex2"
protein A
EcoRI
HindIII

was produced. The *E.coli* strain NM522 was transformed with pKPRA as a control and pKPRAKex: both transformed strains NM522 (pKPRA) and NM522 (pKPRA Kex) were induced to produce the protein A fusion protein (see Materials and Methods). On induction these strains overproduce the protein A fusion proteins to such an extent that insoluble aggregates of the fusion proteins (inclusion bodies) form in the cell. Preparations of inclusion bodies were made (see Materials and Methods) and analysed on Coomassie stained SDS-PAGE (fig. 7.4) and also by Western blotting (fig. 7.4).

The Coomassie stained gel (fig. 7.4) shows quite clearly that a protein is present in the preparation of inclusion bodies from NM522 (pKPRA Kex) that is not present in the preparation of inclusion bodies from NM522 (pKPRA). The protein has an approximate molecular weight of 75K which agrees with the predicted molecular weight of the protein A-Kex2 fusion protein. The same samples analysed by Western blotting (fig. 7.4) and using pre immune sera from rabbit as a source of first antibody, reveals that the protein from the preparation of inclusion bodies from NM522 (pKPRA Kex) contains a protein A moiety that is larger than the protein A containing protein in the preparation of inclusion bodies from NM522 (pKPRA). The protein A containing protein from NM522 (pKPRAKex) is approximately the same size as the predicted protein A-Kex2 fusion protein. Because of the fact that pKPRA Kex appeared to be the correct construct and the fact that a protein A containing fusion protein of the predicted size was produced in NM522 (pKPRA Kex) under inducing conditions, it was assumed that the protein was in fact the protein A Kex2 fusion protein.

7.3 Purification of protein A-Kex2 fusion protein for use as an immunogen

The original intention was to purify the protein A-Kex2 fusion protein from the other proteins in *E.coli* by an affinity purification method using its affinity for IgGs. However other members of the lab experienced problems in affinity purifying protein A fusion proteins for a number of reasons, including the insolubility of the fusion protein inclusion bodies and the co-purification of other proteins with the affinity purified fusion protein. Because of the large amount of protein A-Kex2 fusion protein in inclusion bodies produced in induced cells it was decided to purify

Fig 7.4 Analysis of the preparations of inclusion bodies from NM522 (pKPRA) and NM522 (pKPRA Kex)

The preparations of inclusion bodies from NM522 (pKPRA) and NM522 (pKPRA Kex) were separated on a 10% polyacrylamide gel. Half of the gel was stained with Coommassie blue and the other half containing duplicate samples was blotted onto nitrocellulose. The half blotted onto nitrocellulose was analysed by Western analysis as described in Materials and Methods using pre-immune rabbit serum (1/2000 dilution) as the primary antibody. The Coomassie stained gel (A) and the Western blot (B) are shown. The numbering of the lanes is the same for the Coomassie stained gel and the Western blot.

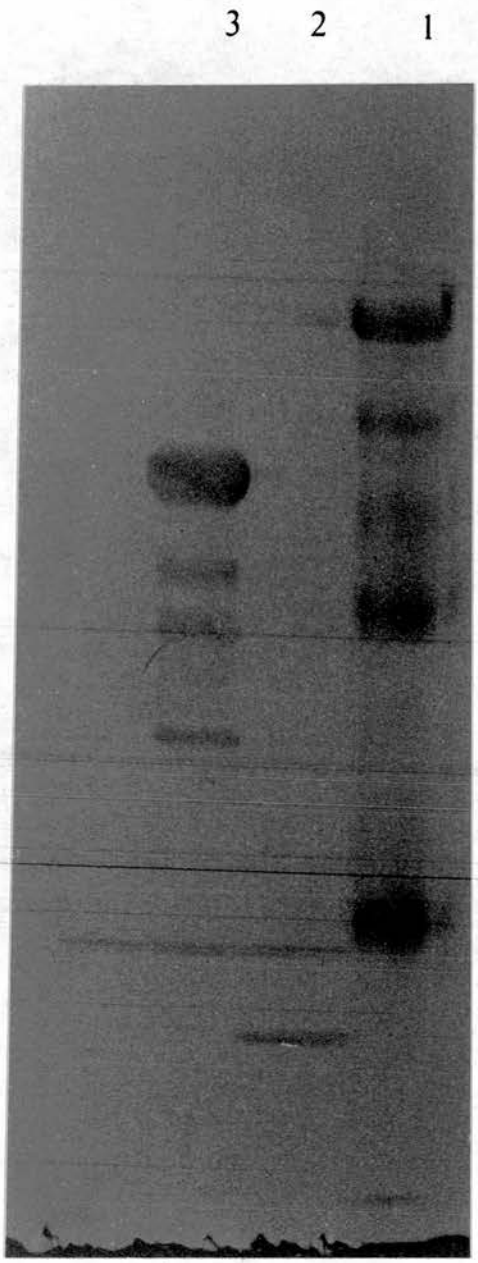
Lane 1. Prestained marker proteins.

Lane 2. Inclusion bodies from NM522 (pKPRA).

Lane 3. Inclusion bodies from NM522 (pKPRA Kex).

A

B



the fusion protein from SDS-PAGE of inclusion body "preps" from induced pKKex transformed cells (see Materials and Methods). The purified fusion protein was analysed by SDS-PAGE and western blotting as before and shown to be the correct protein. The purified fusion protein was used to immunise rabbits and obtain sera hopefully containing antibodies specific to Kex2p (see Materials and Methods).

7.4 Construction of a β-galactosidase-Kex2 fusion protein.

When using polyclonal antiserum it is desirable to be able to purify the antibodies specific to the desired antigen from the other (non specific) antibodies in the serum. This is because the non specific antibodies tend to bind to proteins other than the desired antigen, causing problems in the interpretation of results. This seems to be particularly a problem when studying yeast antigens. In order to be able to affinity purify the antibodies specific for epitopes in Kex2p from other antibodies in the immune sera, we therefore decided to make a β-galactosidase-Kex2 fusion protein. In order to do this the β-galactosidase fusion vector has to contain the same portion of DNA (or part of the same piece of DNA) as pKPRA Kex, in the same reading frame so that the protein A-Kex2 fusion protein and the β-galactosidase-Kex2 fusion protein share Kex2p epitopes.

As with vectors for making protein A fusion proteins, many vectors for making β-galactosidase fusion proteins are also commercially available. Stanley and Luzio (1984) designed vectors (called pEX) that harbour a gene fusion of the *cro* gene of bacteriophage lambda and the *lacZ* gene of *E.coli* that directs the production of a 117kD Cro-β-galactosidase fusion protein. For the purpose of Kex2 fusion an improved plasmid called pEX11 was used (Kusters *et al*) which carries an improved polylinker at the 3' end of the lacZ gene. The plasmids pEX12 and pEX13 along with pEX11, between them allow the fusion of any open reading frame with convenient restriction sites, to be in frame with lacZ and thus produce a fusion protein. Transcription of the hybrid gene of pEX11 is under control of the P_r promoter of bacteriophage lambda. Regulated expression can be achieved in a host strain of *E.coli* that harbours a lysogenised phage lambda carrying the thermosensitive cI_{857} allele (eg pop2136). At 30°C the repressor is functional but at 42°C it is not. Therefore a

shift to 42°C causes the fusion protein to be produced at high levels.

The β-galactosidase-Kex2 hybrid was created by cloning an Eco RI/Bam HI fragment from pKPRAKex into the Eco RI/ Bam HI sites of pEX11 to create pEX Kex (fig. 7.5). This cloning put the ORF of Kex2 in frame with the ORF of β–galactosidase and therefore a β-galactosidase-Kex2 fusion protein should be expressed of expected size 170 K.

The plasmid pEX Kex was transformed into *E.coli* strain pop2136. Transformants were grown in selective media, induced for 4 hours to express fusion protein (see Materials and Methods) and whole cell extracts were made by boiling harvested cells in SDS sample buffer. The whole cell extracts were run on a 10% SDS-PAGE and Western blotted using immune and pre-immune sera from rabbits immunised with the protein A-Kex2 fusion as the source of first antibody.

The immune serum recognised a protein which corresponded to the expected size of the β–galactosidase fusion protein (fig. 7.6) and was not recognised by pre-immune serum. Therefore this protein was assumed to be the β–galactosidase fusion protein. Because the antibodies in the immune serum were raised against protein A-Kex2 fusion protein and the only shared region of the two fusion proteins is the Kex2 portion, the antibodies recognising the β-galactosidase fusion protein were assumed to be Kex2 specific. It was decided to proceed in using the β-galactosidase fusion protein to affinity-purify Kex2p specific antibodies from the immune serum.

7.5 Affinity purification of antibodies

E.coli strain pop2136 tranformed with pEX Kex was grown in selective medium, induced to produce fusion protein and inclusion body preps were made (see materials and methods). The inclusion body prep was ran on a 10% preparative SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose was stained with Ponceau-S and a strip containing the fusion protein, which could be clearly seen (not shown) was cut from the nitrocellulose. This affinity strip was used to affinity purify antibodies specific to Kex2p (see Materials and Methods). The affinity purified antibodies were tested to

Fig. 7.5 Construction of the β-galactosidase-Kex2 fusion plasmid pEX-Kex

For this construction, plasmid pKPRA Kex was used as a source of KEX2 DNA. Plasmid pKPRA Kex was digested with Eco RI and Bam HI and the resulting fragment was purified. Plasmid pEX11 (Kusters et al 1989) was digested with Eco RI and BamHI and was ligated with the Eco RI/Bam HI fragment from pKPRA Kex. This ligation was transformed into pop2136 and transformants harbouring plasmids with the Kex2 insert were selected. The resulting plasmid pEX-Kex encodes a β-galactosidase-Kex2 gene fusion in which the β-galactosidase and Kex2 coding sequences are in frame. The expected size of the fusion protein that should be expressed is 170K.

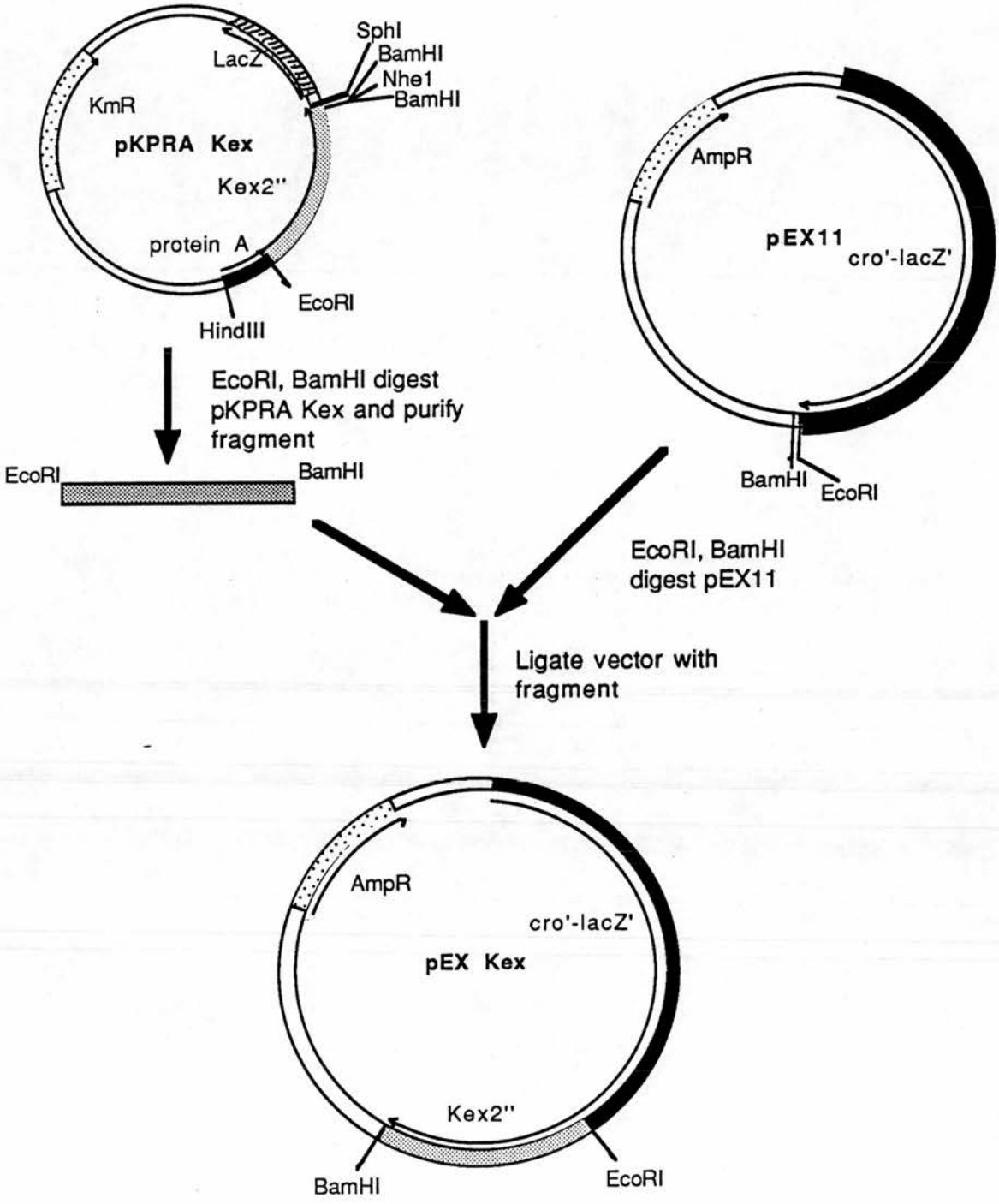

SphI
BamHI
Nhe1
BamHI
LacZ
KmR
pKPRA Kex
Kex2''
protein A
EcoRI
HindIII
EcoRI, BamHI digest
pKPRA Kex and purify
fragment
EcoRI
BamHI
AmpR
pEX11
cro'-lacZ'
BamHI
EcoRI
EcoRI, BamHI
digest pEX11
Ligate vector with
fragment
AmpR
cro'-lacZ'
pEX Kex
Kex2''
BamHI
EcoRI

Fig. 7.6 Analysis of the preparation of inclusion bodies from pop2136 (pEX-Kex).

The preparation of inclusion bodies (duplicate samples) from pop2136 (pEX-Kex) was separated on a 10% polyacrylamide gel. The gel was blotted onto nitrocellulose and analysed by Western analysis using either (A) Kex2-immune serum (anti-serum raised against the protein A-Kex2 fusion protein 1/2000 dilution) or (B) pre immune serum from the same rabbit as the source of primary antibody (1/2000 dilution).

(A) Kex2 immune serum

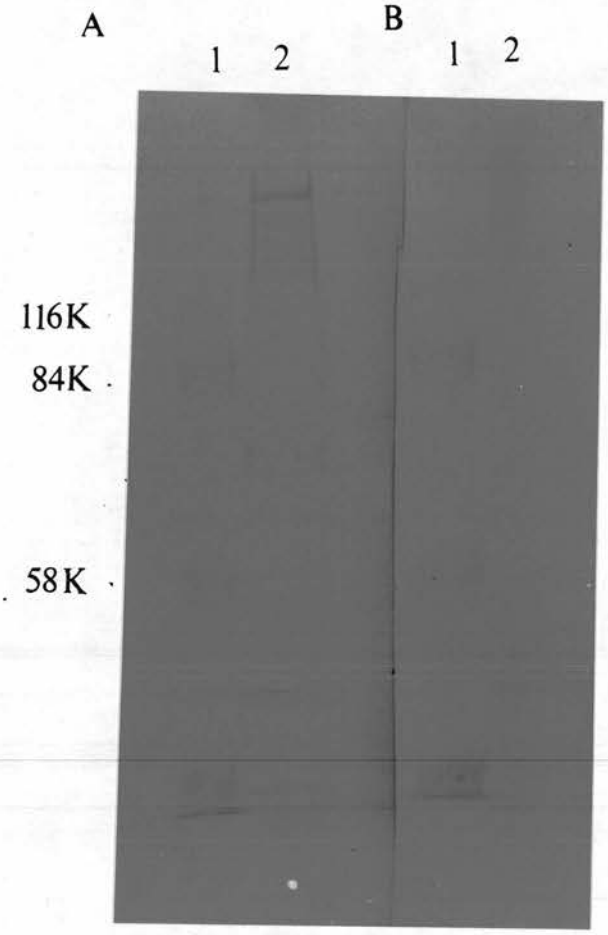
Lane 1. Prestained marker proteins

Lane 2. Inclusion bodies from pop2136 (pEX-Kex)

(B) Pre-immune serum

Lane 1. Prestained marker proteins

Lane 2. Inclusion bodies from pop2136 (pEX-Kex)


A
B
1
2
1
2
116K
84K
58K

see if they would recognise the β-galactosidase fusion protein. The antibodies did recognise the fusion protein and were assumed to be Kex2 specific, as all other antibodies in the serum should have been removed during affinity purification.

In this chapter I have described how anti-Kex2 antibodies were raised and purified using recombinant DNA technology. The use of these affinity purified antibodies will be described in some of the following chapters.

Chapter eight.

Western analysis of intracellular and secreted Kex2-related polypeptides, in strains of yeast expressing different truncated Kex2 polypeptides.

8.0 Introduction

In the previous chapter the production of antiserum against the N-terminal region of Kex2p and the affinity purification of Kex2-specific antibodies from this serum was described. In order to test whether these affinity-purified Kex2 antibodies recognised Kex2 related polypeptides expressed in yeast, I decided to look at the Kex2 polypeptides present in culture supernatants of cells that secreted Kex2p (or at least Kex2 activity) by Western blot analysis. In an initial experiment supernatants from PWYS3 (YEpKS) and PWYS3 (YEpKP) cells grown in selective medium overnight to stationary phase, (which are known to contain Kex2p by the criterion of the in vitro assay for Kex2) were examined by Western blot analysis (see Materials and Methods) using affinity-purified Kex2 antibodies as the first antibody. There were no proteins specifically recognised by the Kex2 antibodies in the supernatants of either of the strains (data not shown). It was presumed that the concentration of Kex2p in the supernatants was too low to allow the protein to be detected by Western blotting, so the supernatants were concentrated and, in addition, crude microsomes were prepared from the two strains in order to attempt to detect the two forms of Kex2' polypeptides expressed in these cells (see Materials and Methods). The concentrated supernatants of were assayed for Kex2 activity and, as expected, the activity was more than 50-fold greater than in the unconcentrated supernatants of each strain (data not shown).

8.1 Western blot analysis of Kex2-related polypeptides in concentrated supernatants and microsomes.

The concentrated supernatants and microsome preparations from PWYS3 (YEpKS) and PWYS3 (YEpKP) were added to equal volumes of protein electrophoresis sample buffer and boiled for 5 minutes. The samples (20 µl) were separated by SDS PAGE on a 10% gel and transferred to nitrocellulose. Western analysis (see Materials and Methods) using affinity-purified Kex2 antibodies as the first antibody was performed to detect Kex2 related polypeptides in the samples.

The result of the Western analysis (fig. 8.1) shows that the proteins recognised by the Kex2 antibodies are different in PWYS3 (YEpKS) and PWYS3 (YEpKP), both in the supernatants and in the preparations of microsomes. This was to be expected as

Fig. 8.1 Western analysis of concentrated supernatants and microsomes of two yeast strains with anti-Kex2 antibodies

The protein in concentrated supernatants and microsomes of strains PWYS3 (YEpKS) and PWYS3 (YEpKP) were separated on 10% SDS PAGE and transferred to nitrocellulose. The nitrocellulose blot was probed with affinity purified anti-Kex2 antibodies (1/100 dilution) and the proteins recognised by the antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (The ECL method of detection was used as described in materials and methods). The marker proteins in this experiment and all the following Western analyses are a mixture of protein A fusion proteins of different sizes made in our laboratory (Dr. J. Zueco)

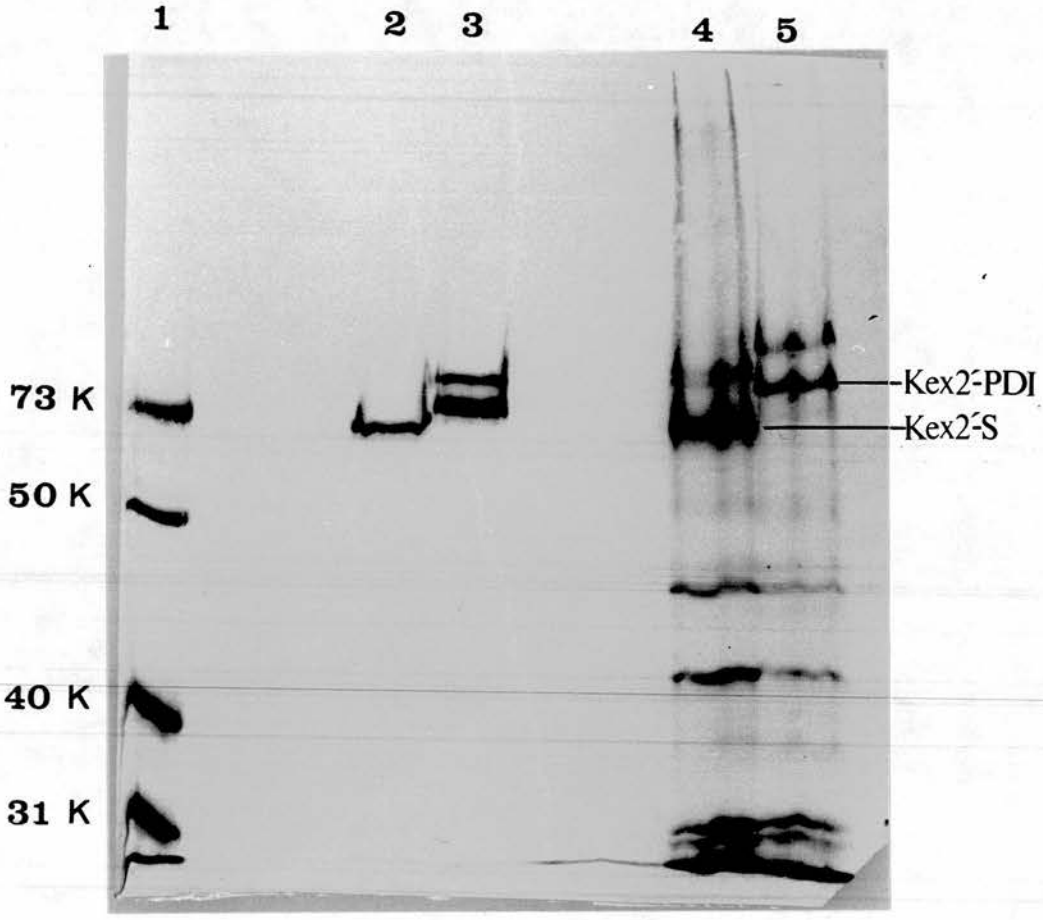
Lane 1. Marker proteins

Lane 2. PWYS3 (YEpKS) concentrated supernatant.

Lane 3. PWYS3 (YEpKP) concentrated supernatant.

Lane 4. PWYS3 (YEpKS) microsomes.

Lane 5. PWYS3 (YEpKP) microsomes.


1
2
3
4
5
73 K
50 K
40 K
31 K
Kex2-PDI
Kex2-S

the polypeptides supposedly expressed from these strains, Kex2'-S and Kex2'-PDI have predicted molecular weights of 70K and 76K respectively. This result therefore provided further evidence that the antiserum raised against the protein-A-Kex2 fusion protein, and the antibodies affinity purified against the β-galactosidase-Kex2 fusion protein were in fact Kex2-specific. Without this information, only the fact that the crude antiserum recognised the β-galactosidase-Kex2 fusion protein had provided evidence that the antibodies in the immune serum were Kex2-specific. Also the result showed that Kex2' polypeptides expressed in *S. cerevisiae* could be recognised by the affinity purified antibodies. As well as confirming the specificity of the antibodies the fact that Kex2-related polypeptides of different molecular weights were recognised in PWYS3 (YEpKS) and PWYS3 (YEpKP) provided evidence that the expected Kex2 polypeptides (ie Kex2'-S and Kex2'-PDI) were being expressed in their respective strains (the only previous evidence was that the YEpKS and YEpKP constructs were correct as far as it was possible to tell).

One observation from the blot was that there appears to be some heterogeneity of polypeptides expressed in the individual cell supernatants and preparations of microsomes. It is known that the portion of Kex2p in the Kex2' constructs contains a single N-linked glycosylation group (Fuller *et al* 1989) and therefore this heterogeneity was thought possibly to reflect different glycosylation states of the Kex2' polypeptides. This possibility was examined by treating all of the samples with endoglycosidase H (Endo H) to remove the N-linked carbohydrate and comparing the treated samples with untreated samples by Western analysis (fig. 8.2). If the heterogeneity was due to different N-linked glycosylation states of the Kex2' polypeptides we would expect that on treatment with Endo H, the Kex2' polypeptides would migrate as a single protein band on the blot, corresponding to their unglycosylated forms. The results of the Endo H treatment reveal that the heterogeneity of polypeptides recognised by Kex2 antibodies in individual samples is difficult to explain by differences in N-glycosylation alone: treatment of the supernatant of PWYS (YEpKS) with Endo H causes a shift in mobility of the Kex2 related polypeptide. This is consistent with a single N-linked carbohydrate being removed from Kex2'-S. However if this is the case, it is difficult to account for the slower migrating (and less abundant) polypeptides recognised by Kex2 antibodies in the microsomes of PWYS3 (YEpKS) by differences in N-linked glycosylation. In fact

Fig 8.2 Western analysis of endoglycosidase H-treated supernatants and microsomes of two yeast strains using anti-Kex2 antibodies.

The Endoglygosidase H (Endo H)-treated and untreated protein samples (the same samples as in fig. 8.1) were separated by 10% SDS PAGE and transferred to nitrocellulose. The nitrocellulose blot was probed with affinity-purified anti Kex2 antibodies (1/100 dilution) and the proteins recognised by the antibodies were detected using horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibodies (The ECL method of detection was used as described in materials and methods).

Lane 1. Marker proteins

Lane 2. PWYS3 (YEpKS) concentrated supernatant (untreated)

Lane 3. PWYS3 (YEpKS) concentrated supernatant (Endo H treated)

Lane 4. PWYS3 (YEpKP) concentrated supernatant (untreated)

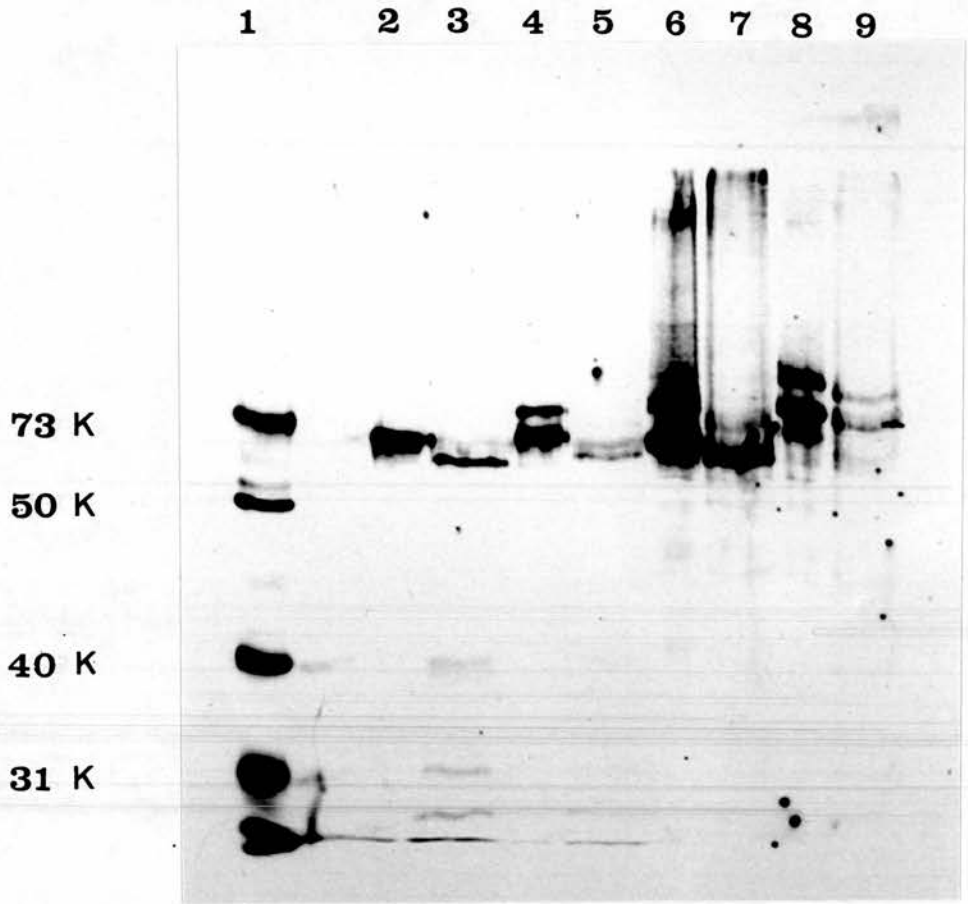
Lane 5. PWYS3 (YEpKP) concentrated supernatant (Endo H treated)

Lane 6. PWYS3 (YEpKS) microsomes (untreated)

Lane 7. PWYS3 (YEpKS) microsomes (Endo H treated)

Lane 8. PWYS3 (YEpKP) microsomes (untreated)

Lane 9. PWYS3 (YEpKP) microsomes (Endo H treated)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6
7
8
9
73 K
50 K
40 K
31 K

it is difficult to account the slower migrating forms of Kex2 related protein at all, as the fastest migrating form of Kex2-related protein recognised in the microsomes has a change in mobility when treated with Endo H which suggests that it is N-glycosylated. I have therefore assumed that this most abundant and fastest migrating species of polypeptide in the microsomes of PWYS3 (YEpKS) is the Kex2'-S polypeptide with a single N-linked glycosylation group (marked on fig. 8.1) (the reason for this assumption will be discussed later in the chapter). Thus a possible explanation for polypeptides larger than singly N-glycosylated Kex2'-S being present is that they are Kex2'-S polypeptides containing additional post translational modifications such as O-linked glycosylation.

In the microsomes of PWYS3 (YEpKP), I have assumed again, that the smaller, more abundant polypeptide is Kex2'-PDI that is contains a single N-glycosyl group (marked on fig. 8.1), as it has an altered mobility after treatment with Endo H (fig, 8.2); and that the larger, less abundant forms have some additional post translational modification. An interesting observation is that the major secreted polypeptide from PWYS3 (YEpKP) appears to be smaller than the intracellular polypeptide that is assumed to be singly N-glycosylated Kex2'-PDI. This difference cannot be explained by differences in N-glycosylation as the major secreted polypeptide also appears to be N-glycosylated as it has an altered mobility after treatment with Endo H.

The observation that the major secreted form of Kex2 related polypeptide from PWYS3 (YEpKP) is smaller (by approximately 4K) than the major intracellular form (Kex2'-PDI) is confirmed in fig. 8.3. The major forms of secreted and intracellular Kex2-related protein expressed in PWYS3 (YEpKS), however, have identical apparent molecular weights (approximately 70K; fig. 8.3). This agrees with the predicted molecular weight of Kex2'-S, therefore these forms were assumed actually to be Kex2'-S (singly N-glycosylated as shown by the Endo H experiments; this was also why the larger forms of Kex2 related protein detected in the microsomes were assumed to have some additional post translational modification). One explanation for the secreted polypeptide from PWYS3 (YEpKP) being approximately 4 K smaller than the intracellular Kex2'-PDI polypeptide is that it might be a proteolytic cleavage product of Kex2'-PDI (called Kex2'-PDI'). One piece of evidence for this is that the polypeptide secreted from PWYS (YEpKP) is not

Fig. 8.3 Western analysis to compare the secreted and intracellular forms of Kex2p produced in two different strains of yeast using anti-Kex2 antibodies.

The proteins in concentrated supernatants and microsomes from PWYS3 (YEpKS) and PWYS3 (YEpKP) (the same samples as in fig 8.1) were separated on 10% SDS PAGE and transferred to nitrocellulose. Supernatants and microsomes from each of the strains were loaded in adjacent lanes so that secreted and internal proteins could be compared. The nitrocellulose blot was probed with affinity-purified anti-Kex2 antibodies (1/100 dilution) and the proteins recognised by the antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti rabbit IgG antibodies (The ECL method of detection was used as described in materials and methods).

Lane 1. Marker proteins.

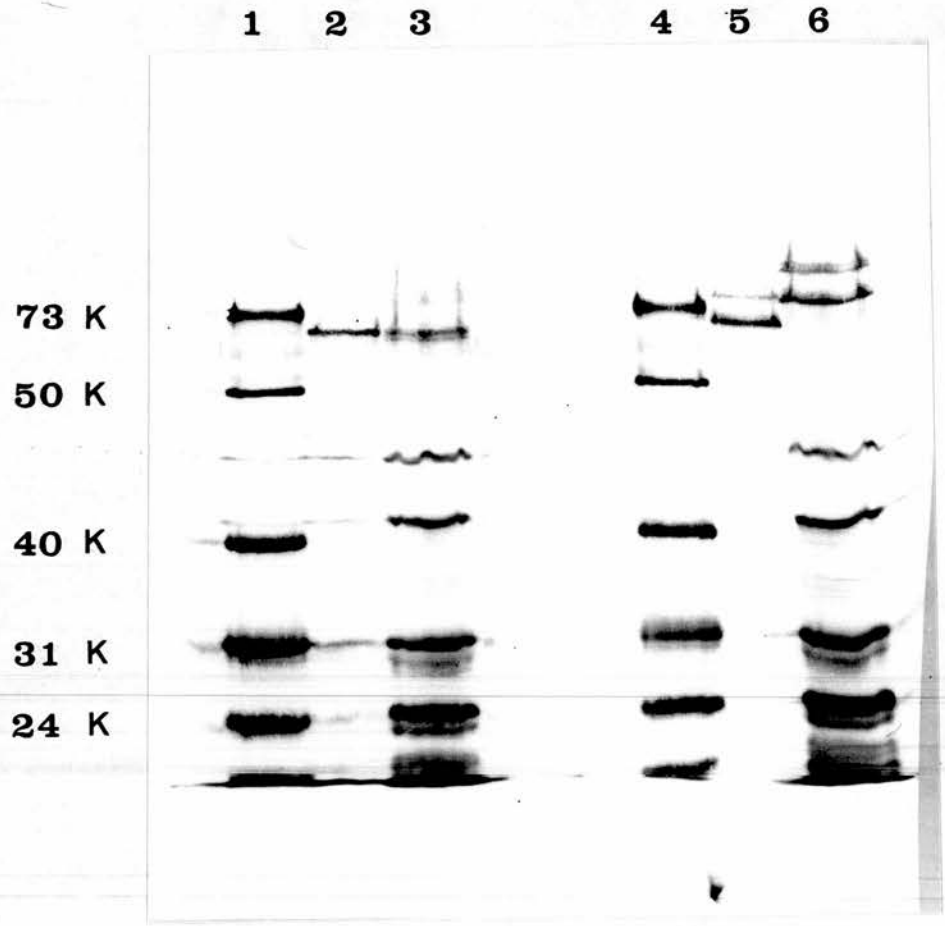
Lane 2. PWYS3 (YEpKS) concentrated supernatant (secreted).

Lane 3. PWYS3 (YEpKS) microsomes (internal).

Lane 4. Marker proteins.

Lane 5. PWYS3 (YEpKP) concentrated supernatant (secreted).

Lane 6. PWYS3 (YEpKP) microsomes (internal).


1
2
3
4
5
6
73 K
50 K
40 K
31 K
24 K

recognised by HDEL antibodies (fig. 8.4) and Kex2'-PDI should have the HDEL signal at its extreme C-terminus. As this putative cleavage does not apparently occur in secreted Kex2'-S, it seems reasonable to assume that the cleavage occurs in the PDI portion of Kex2'-PDI. Evidence for this is that the 60 C-terminal amino acids of PDI in Kex2'-PDI is the only region of the polypeptide not identical with Kex2'-S and if the putative cleavage were to occur in the common Kex2' portion of Kex2'-PDI we would also expect it to occur in Kex2'-S. Also, if this putative cleavage had occurred in the Kex2' portion of Kex2'-PDI, the polypeptide secreted would be smaller than Kex2'-S; this is not the case and Kex2'-PDI' is larger than secreted Kex2'-S (fig. 8.1). Autoproteolytic cleavage by Kex2 has been ruled out as there are no Kex2 cleavage sites in the PDI portion of Kex2'-PDI.

If cleavage in the PDI portion of Kex2'-PDI is the explanation for the smaller secreted Kex2'-PDI' polypeptide, a number of results in previous chapters become easier to explain. Kex2'-PDI' would not contain the HDEL sequence or a substantial portion of the PDI C-terminus as Kex2'-PDI' is approximately 4K smaller than Kex2'-PDI. This would explain the reason for Kex2 activity being secreted into the growth medium of PWYS3 (YEpKP) even though Kex2'-PDI was expected to be efficiently retained in the cell. Although Kex2'-PDI is the most efficiently retained fusion protein, cleavage of a 4K portion from the C-terminus would be expected to abolish retention due removal of the HDEL signal and amino acids of the C-terminus of PDI.

8.2 Western analysis of HDEL-containing proteins in concentrated supernatants and microsomes

Kex2'-PDI and Kex2'-HDEL should both have the C-terminal HDEL sequence whereas Kex2'-S should not. Microsomes and concentrated supernatants from PWYS3 (YEpKP), PWYS3 (YEpKH) and PWYS3 (YEpKS) were prepared as before (see Materials and Methods). To detect proteins containing the HDEL sequence at the C-terminus we decided to use anti-HDEL antibodies (obtained from Dr. H. Pelham) for Western analysis of the samples from the three strains (fig. 8.4). Western analysis of the same samples was also performed using the affinity purified anti-Kex2 antibodies, and the results compared with those obtained using the anti-HDEL antibodies (fig. 8.4).

Fig. 8.4 Western analysis of concentrated supernatants and microsomes of three yeast strains using anti-HDEL and anti-Kex2 antibodies

The proteins in concentrated supernatants and microsomes of strains PWYS3 (YEpKH), PWYS3 (YEpKS) and PWYS3 (YEpKP) were separated on 10% SDS PAGE and transferred to nitrocellulose (identical samples were loaded onto two gels). The nitrocellulose blots were probed with either rabbit anti-HDEL antiserum (A) (obtained from Dr H. Pelham) or affinity purified anti-Kex2 antibodies (B), and the proteins recognised by the antibodies were detected using horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibodies (The ECL method of detection was used as described in materials and methods).

Lane 1. Marker proteins

Lane 2. PWYS3 (YEpKH) microsomes.

Lane 3. PWYS3 (YEpKS) microsomes.

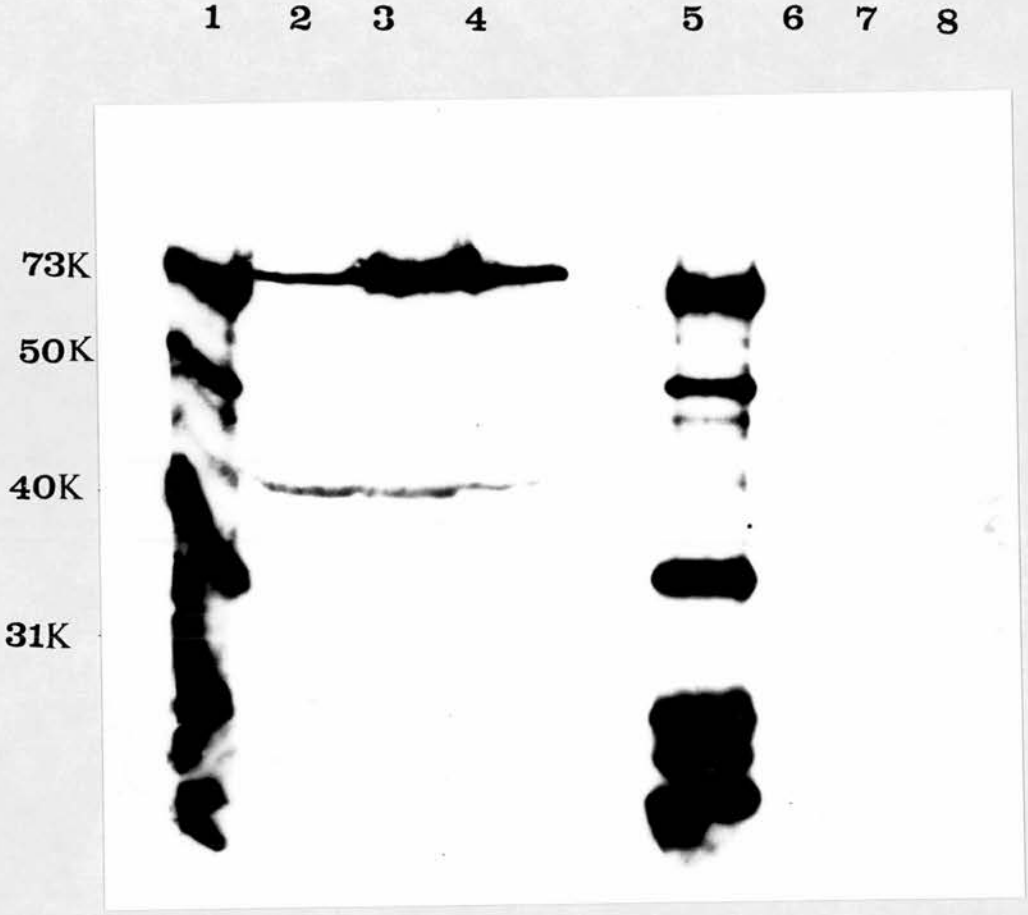
Lane 4. PWYS3 (YEpKP) microsomes.

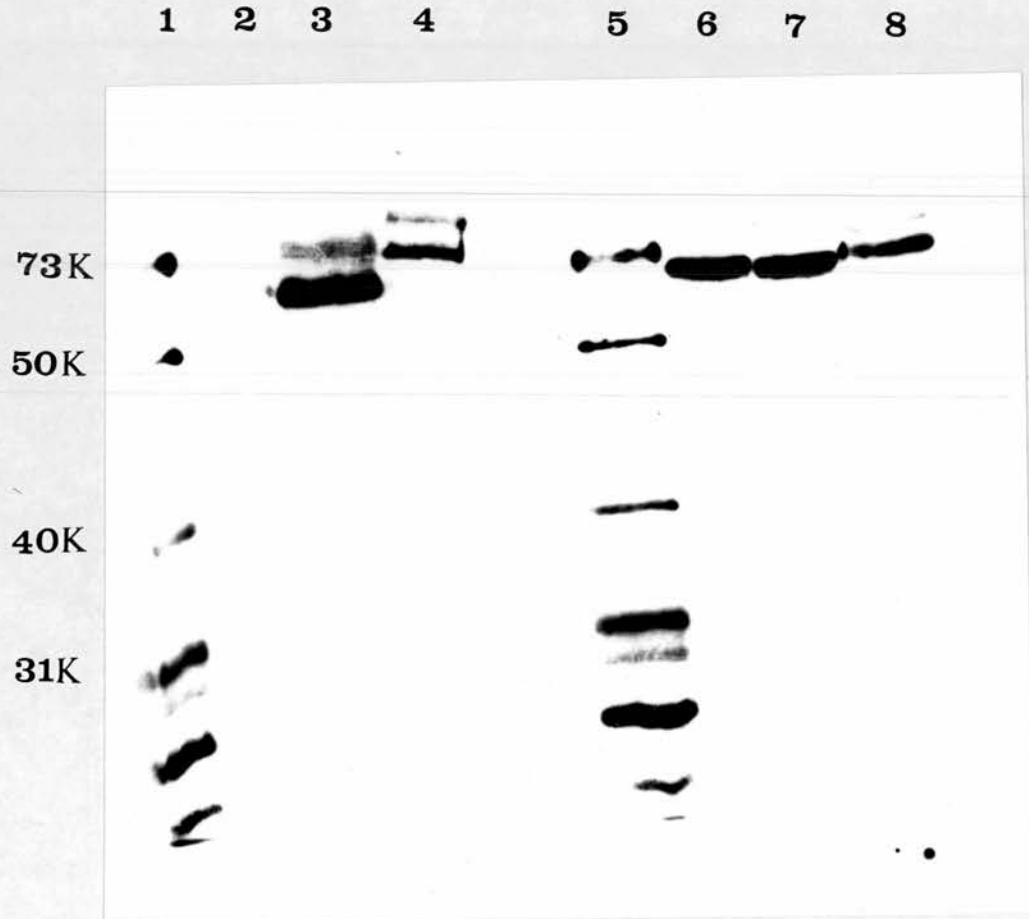
Lane 5. Marker proteins.

Lane 6. PWYS3 (YEpKH) concentrated supernatant.

Lane 7. PWYS3 (YEpKS) concentrated supernatant.

Lane 8. PWYS3 (YEpKP) concentrated supernatant.


1 2 3 4 5 6 7 8
73K
50K
40K
31K


1 2 3 4 5 6 7 8
73K
50K
40K
31K

There are no proteins recognised by the HDEL antibodies in the culture supernatants of any of the three strains. In the microsomes prepared from the three strains however, two proteins were recognised by the HDEL antibodies (fig. 8.4). As these recognised proteins are the same size in each strain and we know that Kex2'-PDI is larger than Kex2'-HDEL and Kex2'-S (fig. 8.4) it is unlikely that the proteins recognised by the HDEL antibodies are Kex2 -related. It is likely that the protein with the approximate molecular weight of 73K is yeast BiP (Rose *et al* 1989) and it is possible that the protein with the approximate molecular weight of 45K is yeast PDI (although the predicted molecular weight of yeast PDI from its DNA sequence is 58K; Dr. M. Tuite, personal communication). Thus it appears that the Kex2' polypeptides expressed in these strains are not detectable with anti-HDEL antibodies even though they are recognised by anti-Kex2 antibodies. As both of the Western blots (figs. 8.4) were identical with respect to the samples loaded, these results suggest that the anti-HDEL antibodies may not have such a high affinity for their antigen as the anti-Kex2 antibodies do for their antigen. Therefore the anti-HDEL antibodies recognise only abundant proteins (ie BiP) and did recognise Kex2' polypeptides (Kex2'-HDEL or Kex2'-PDI) as they are in too small amounts to be detectable. The anti-Kex2 antibodies may be more efficient at recognising their antigen because of their ability to recognise several epitopes in Kex2' and thus recognise Kex2' polypeptides in amounts that cannot be detected with the anti-HDEL antibodies. As it was not possible to detect Kex2'-HDEL or Kex2'-PDI in either microsomes or supernatants it is not possible to test the idea that the HDEL sequence is not present on secreted Kex2'-PDI' using the HDEL antibodies. Thus although we suspect that the HDEL sequence has been removed along with some of the PDI sequence of Kex2'-PDI to yield Kex2'-PDI' it is difficult to prove with the resources available. The possibility cannot be ruled out that Kex2'-HDEL has the HDEL signal removed before being secreted although this is less likely as the intracellular and secreted forms of Kex2' polypeptide expressed in PWYS3 (YEpKH) are of the same apparent molecular weights.

Chapter nine.

Immunolocalisation of the different truncated Kex2 polypeptides by immunogold labelling of yeast cells.

9.0 Introduction.

From the results described in previous chapters it appears that the addition of the different C-termini to a truncated Kex2p has an effect on the efficiency of retention within the cell. The addition of the 60 C-terminal amino acids of yeast PDI to the C-terminus of the truncated Kex2p to create a Kex2'-PDI fusion protein appears to result in efficient retension of this protein although some Kex2'-PDI hybrid protein is secreted in an apparently cleaved form. Although it is known that there are internal Kex2-related proteins (shown by Western analysis) and Kex2 activity (shown by *in vitro* Kex2 assays) in the strains expressing the truncated Kex2 proteins with the different C-termini, the exact location of these proteins within the cell is not known. To investigate the distribution of the Kex2' polypeptides within the yeast cell, it was decided to attempt immunogold labelling of the strains. The affinity-purified anti-Kex2 antibodies appeared to be specific for Kex2 related proteins (shown by Western analysis) and therefore these antibodies were used in an attempt to specifically label Kex2-related proteins in immunogold labelling experiments. The following strains were prepared for immunogold labelling by the methods described in Chapter2 (Materials and Methods):- JRY 188, PWYS3, PWYS3 (YEpKH), PWYS3 (YEpKK), PWYS3 (YEpKS) and PWYS3 (YEpKP). The reason for using the episomal plasmid bearing strains was that these strains express more Kex2'p and therefore detection of the Kex2' polypeptides should be made easier.

9.1 Results of immunolabelling experiments.

In order to be convinced that results of immunolabelling experiments are real and not artefactual it is necessary to carry out as many controls as possible. The first control used here was to omit the primary antibody from the immunolabelling procedure (ie replace first antibody solution with blocking solution) in order to determine whether the gold labelling in the cells is dependent on the presence of the first antibody. If the gold labelling is not dependent on the first antibody being present the labelling must be non specific. Strain JRY 188 was treated as usual for immunolabelling except that the first antibody solution was replaced with blocking solution. The electron micrograph in fig 9.1 shows a typical cell from this experiment. There is virtually no gold labelling in this cell which suggests that any gold labelling in other strains is most probably due to the specificity of the first

antibody. A no first antibody control was performed for all the strains with the same result (ie virtually no labelling with gold. Data not shown).

The second control was performed in order to determine whether labelling when first antibody is present is only in strains expressing Kex2p in some form. Strain PWYS3 does not contain any Kex2p due to the *KEX2* gene being disrupted. Thus any labelling of this strain with gold is not due to antibody recognising Kex2p but due to non specific labelling. Strain PWYS3 was treated as described for immunolabeling with anti-Kex2 antibodies. The electron micrograph in fig 9.2 shows a typical cell from this experiment. There is virtually no labelling of PWYS3 which suggests that gold labelling in other experiments is probably due to labelling of Kex2p and not to non specific labelling. Thus these two controls suggest that gold labelling is due to a specific labelling of Kex2p by the affinity purified anti-Kex2 antibodies.

Strain JRY188 expresses the full length wild type Kex2p that is reportedly localised in the Golgi apparatus. Typical results of immunolabelling of this strain with anti-Kex2 antibodies are shown in fig 9.3 and fig 9.4. Labelling with gold appears to be dispersed throughout the cell and not localised in a particular area. The yeast Golgi apparatus is not well-defined cytologically especially in wild type cells and therefore it is not possible to say whether the labelling is typical of a yeast Golgi protein or not.

Typical results of immunolabelling with anti-Kex2 antibodies of the episomal plasmid bearing strains expressing the truncated Kex2p's with the different C-termini are shown in fig. 9.5 {PWYS3 (YEpKH)}, fig. 9.6 {PWYS3 (YEpKK)}, fig. 9.7 {PWYS3 (YEpKS)}, figs 9.8 and 9.9 {PWYS3 (YEpKP)}. Labelling of all of these strain with gold also appears to be dispersed throughout the cell as with JRY 188. It is not possible to say whether the gold labelling is associated with particular organellar structures as we had hoped would be the case. Large clumps of gold particles are common in strains PWYS3 (YEpKS) and PWYS3 (YEpKP) but less common in strains PWYS3 (YEpKH) and PWYS3 (YEpKK).

The centromere plasmid bearing strains of yeast:- PWYS3 (YCpKH), PWYS3 (YCpKK), PWYS3 (YCpKS) and PWYS3 (YCpKP) were processed for immunocytochemistry as descibed and immunolabelled with anti-Kex2 antibodies the

Figs. 9.1-9.9 Electron micrographs of different strains of yeast immunolabelled with anti-Kex2 antibodies.

All strains of yeast were processed for immunocytochemistry with LR white resin as described in Materials and Methods. The strains were typically immunolabelled as described in this Materials and Methods with anti-Kex2 antibodies (1/50 dilution) as the primary antibody, although a control in which primary antibody was omitted is also shown. The electron micrographs were all taken at a magnification of x17000.

(9.1) JRY 188 (no primary antibody)

(9.2) JRY kex2::URA3-1320 (anti-Kex2 antibody 1/50 dilution)

(9.3) JRY 188 (anti-Kex2 antibody 1/50 dilution)

(9.4) JRY 188 (anti-Kex2 antibody 1/50 dilution)

(9.5) JRY kex2::URA3-1320 (YEpKH) (anti-Kex2 antibody 1/50 dilution)

(9.6) JRY kex2::URA3-1320 (YEpKK) (anti-Kex2 antibody 1/50 dilution)

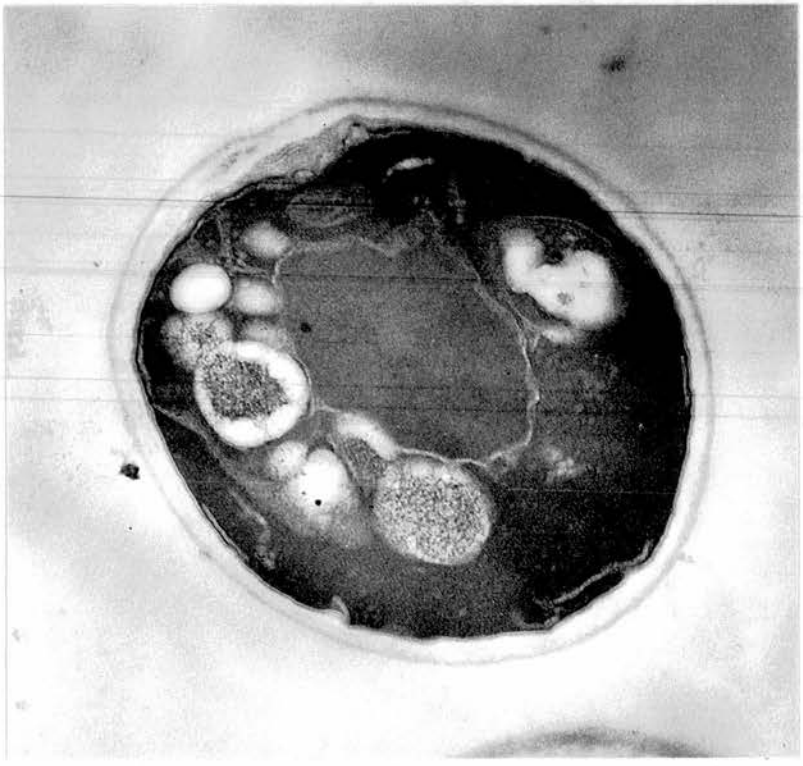
(9.7) JRY kex2::URA3-1320 (YEpKS) (anti-Kex2 antibody 1/50 dilution)

(9.8) JRY kex2::URA3-1320 (YEpKP) (anti-Kex2 antibody 1/50 dilution)

(9.9) JRY kex2::URA3-1320 (YEpKP) (anti-Kex2 antibody 1/50 dilution)

9·1

9·2



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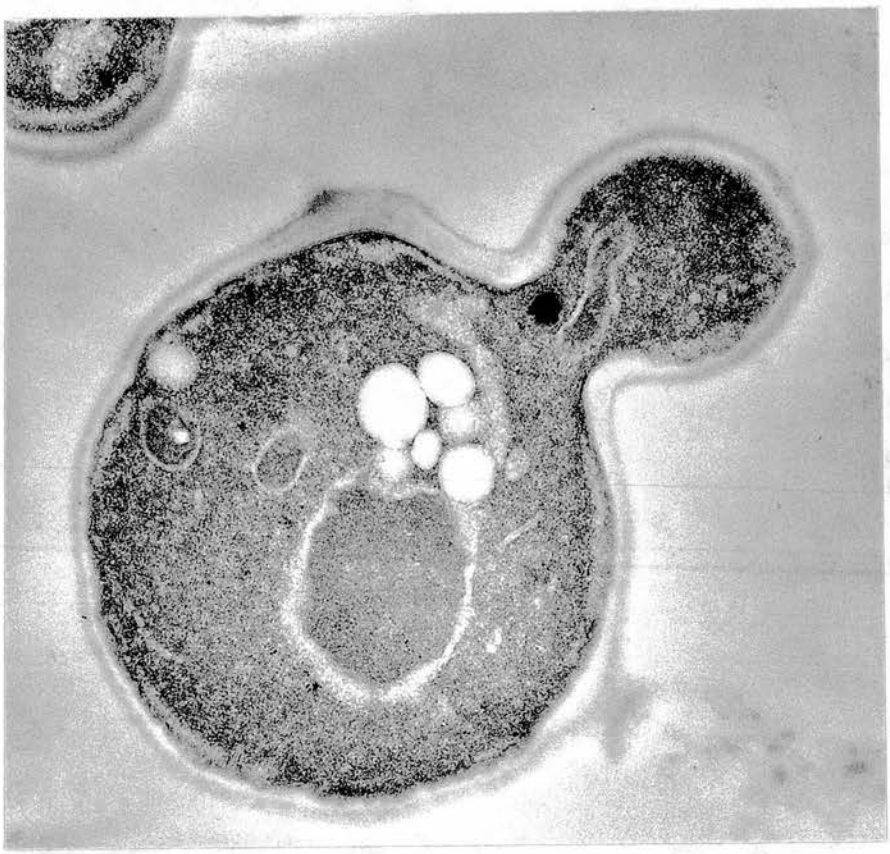

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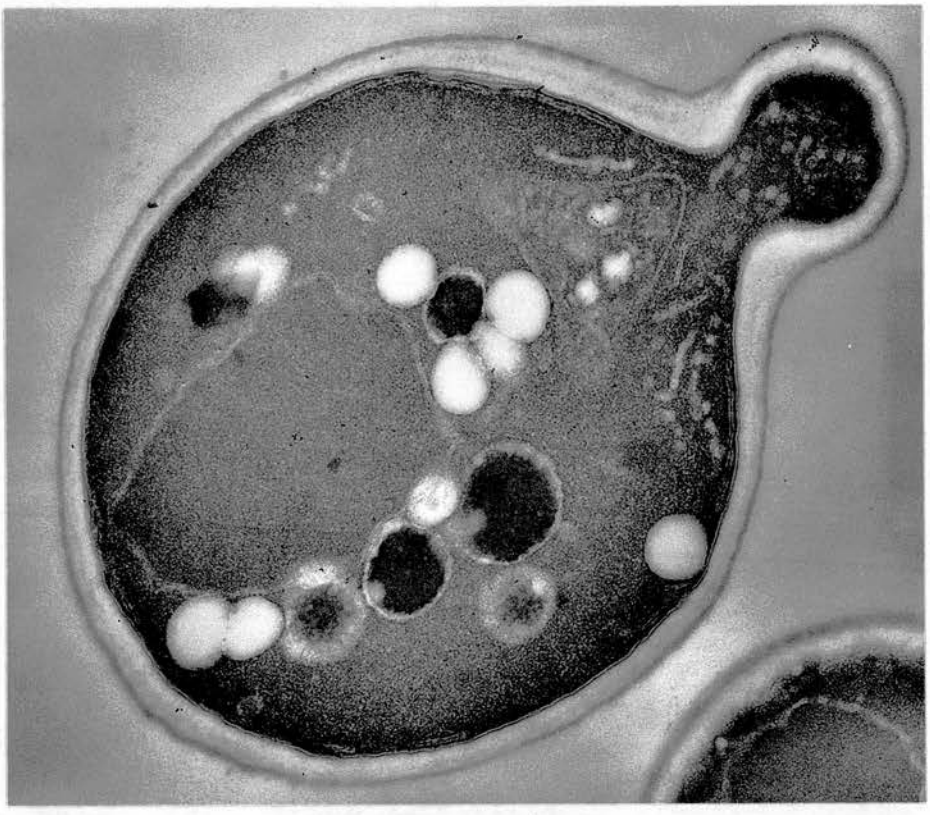

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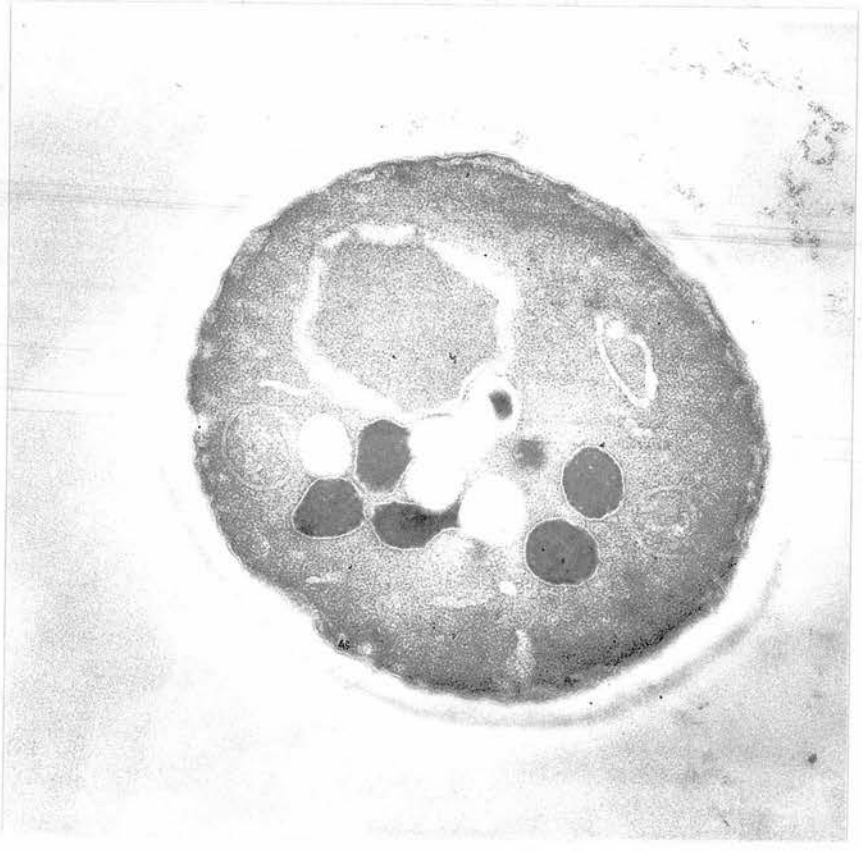

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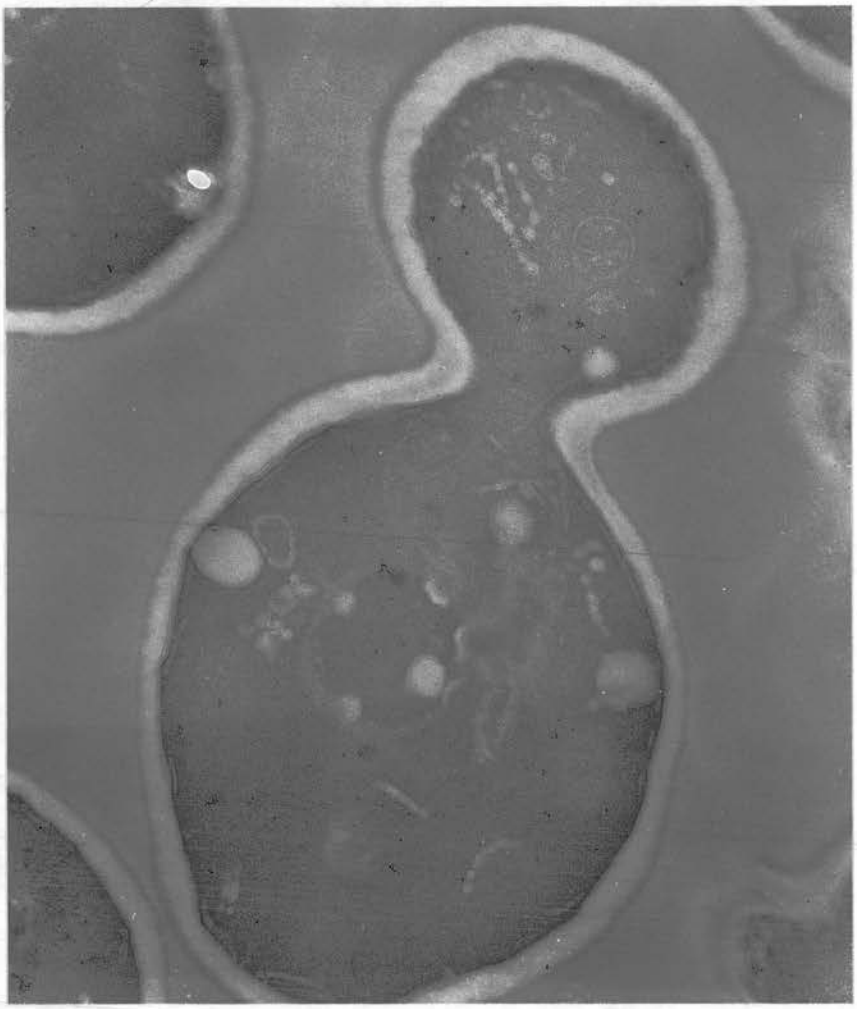

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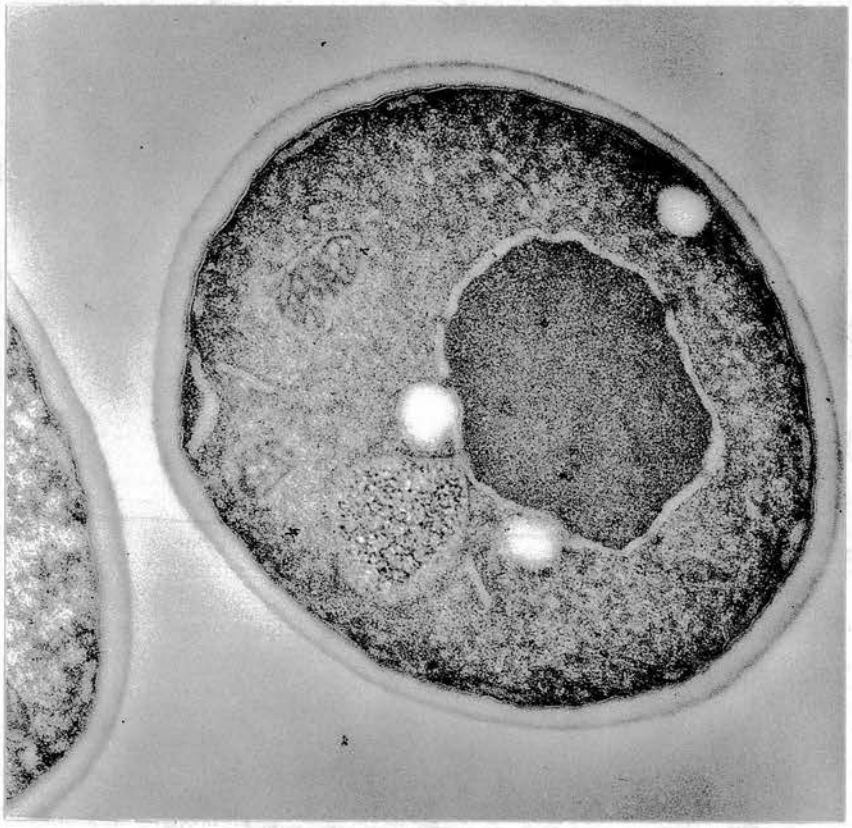


9·9

same as the episomal plasmid bearing strains. There was very little labelling of any of the centromere plasmid bearing strains expressing the Kex2' polypeptides with the different C-termini (Data not shown).

9.3 Conluding remarks.

Labelling in the centromere plasmid bearing strains expressing the Kex2 polypeptides with the different C-termini is much lower than in the episomal plasmid bearing strains which contain the most label. The controls ie when first antibody is omitted and labelling of strain PWYS3 contain virtually no gold label. Thus it appears the cells expressing the most Kex2'p are labelled to the greatest extent and labelling of cells expressing lower amounts of Kex2'p contain virtually no label. Therefore the majority of gold labelling seems to be due to recognition of Kex2p by the first antibody (anti-Kex2 antibody). Unfortunately however, it is difficult to interpret the results of the immunolabelling experiments with any great confidence as the amount of gold labelling even in the episomal plasmid-bearing strains is not very high (although it is higher than in other strains) and does not appear to be well localised.

There are no striking differences in the labelling of any of the episomal plasmid bearing strains as we may have expected from the differences in intracellular Kex2 activity, especially between PWYS3 (YEpKP) which has by far the greatest intracellular Kex2 activity and the other episomal plasmid bearing strains which have less intracellular Kex2 activity (Chapter 7). Thus although labelling appears specific to cells expressing Kex2p at relatively high levels in comparison (the levels of Kex2p in the cell are probably very low in relation to total protein content of the cell) nothing can be deduced about the location of the Kex2p's in the different strains. It is possible that the distribution of gold in the cell corresponds to the subcellular distribution of the different Kex2' polypeptides but it is also possible that the results are artefactual due to the methods used to process the cells. Clearly these results are somewhat disappointing and of a preliminary nature: they are included here for the sake of completeness.

Chapter ten

Biogenesis of the *K.lactis* killer toxin processed by the different Kex2 polypeptides.

10.0 Introduction

In previous chapters the effect of the different Kex2p constructs on the processing of α factor has been analysed. One of my aims at the start of this project was to investigate the biogenesis of the *K.lactis* killer toxin in the secretory pathway of *S.cerevisiae*. The Kex2p has been shown to be involved in the maturation of the of the αβ precursor polypeptide into the mature α and β subunits of the toxin most probably at a late stage in the secretory pathway after the *sec18* mutation exerts its effects (ie in the Golgi complex). It was therefore of interest to investigate the biogenesis of the toxin when processed by the different Kex2p constructs, some of which possibly process in the ER. It was proposed to see if functional killer toxin could be assembled and secreted from strains expressing the different Kex2 constructs and if not the reason for this (ie the Kex2p may not process the αβ precursor, or if it does, processing at an earlier stage in the secretory pathway may not allow the toxin subunits to assemble correctly).

In order to study the effect of the different Kex2 constructs on the biogenesis of the *K.lactis* killer toxin a suitable strain expressing the toxin had to be used. The strain would have to have the chromosomal KEX2 gene inactivated and the plasmids encoding the different Kex2' constructs transformed into it. The only strain expressing the toxin and possessing auxotrophic markers with which to select transformants was F102-2 (k1, k2, *leu2*, *his4*). To inactivate the chromosomal KEX2 gene the only method available was to replace it with a disrupted Kex2 gene (ie *kex2::LEU2* from plasmid pGA1070 as in Chapter 1). The problem with this was that the resulting strain would then only possess auxotrophy for histidine. The plasmids for the expression of the different Kex2' constructs are all *TRP1* plasmids and it would not therefore be possible to select for transformants in F102-2 disrupted with *kex2::LEU2*. Although it is possible to move the DNA encoding the different Kex2' constructs into other plasmids quite easily, suitable plasmids containing the *HIS4* gene as selectable marker in yeast do not exist. The possibility of creating a yeast strain possessing the correct auxotrophies and the linear plasmids k1 and k2 was also ruled out as standard crossing procedures of ρ^o strains of *S.cerevisiae* to create new strains is not possible (ρ^o diploids do not sporulate, and the linear plasmids are only stable in ρ^o strains of *S. cerevisiae*). It was therefore decided to disrupt the

TRP1 gene of F102-2 in order to make it Trp$^-$ so that TRP plasmids can be selected for in transformations.

10.1 Disruption of the chromosomal *TRP1* gene of F102-2

In order to disrupt the chromosomal copy of the *TRP1* gene, a plasmid was contructed in which *TRP1* or at least part of the *TRP1* gene is disrupted. This disrupted gene should be able to be excised as a linear DNA fragment from its vector and transformed into F102-2 to disrupt the chromosomal *TRP1* gene by homologous recombination. A portion of the *TRP1* gene from YRp7 was cloned into pUC9 (fig. 10.1). The resulting plasmid was named pUC-trp'. The *TRP1* portion of pUC-trp' was disrupted with a fragment of bacteriophage λ DNA resulting in a plasmid called pUC-trp'::λ (fig. 10.2). The linear fragment containing the portion of *TRP1* disrupted with λ DNA was excised from pUC-trp'::λ and used in co-transformation of F102-2 with the linear piece of DNA from pGA1070 containing the *kex2::LEU2* disruption (see fig. 3.1). Initially Leu$^+$ transformants were selected for on SD + HW plates. Tryptophan was added to the plates so that as well as the Leu$^+$ transformants being able to grow, any cells that had been made Trp$^-$ due to integration of the disrupted portion of the *TRP1* gene would also grow. Thus the Leu$^+$ transformants selected should be Kex2$^-$ due to integration of the linear DNA encoding the kex2::LEU2 disruption but a mixture of Trp$^+$ and Trp$^-$ colonies. These Leu$^+$ transformants were screened by replica plating onto SD + H and SD +HW plates and transformants that were Trp$^-$ were selected (ie would grow on SD + HW but not SD + H). Thus a strain based on F102-2 that was Kex2$^-$ and also Trp$^-$ was made. This strain could be transformed with the TRP plasmids encoding the different Kex2' constructs. The strain was named PWYS4.

10.2 Analysis of toxin production in strains expressing the different Kex2p constructs

The strain PWYS4 was transformed with the plamids YCpKH, YCpKK, YCpKS and YCpKP and Trp$^+$ transformants were selected. The resulting strains were called PWYS4 (YCpKH), PWYS4 (YCpKK), PWYS4 (YCpKS) and PWYS4 (YCpKP) respectively. An initial experiment on the strain PWYS4 and the transformed strains

Fig. 10.1 Construction of plasmid pUC-trp'

The Eco RI fragment (approximately 1.45 kb) from plasmid YRp7 (Parent *et al* 1986) containing the full length *TRP1* gene is shown. The Eco RI/Hind III fragment from YRP7 coding for a portion of *TRP1* (trp') was ligated with plasmid pUC9 that had been digested with Eco RI/Hind III, and the ligation was transformed into NM522. White colonies were selected on X-Gal plates, and recombinant plasmids were screened by restriction analysis for the presence of the Eco RI/Hind III fragment from YRP7. The resulting plasmid was named pUC-trp'.

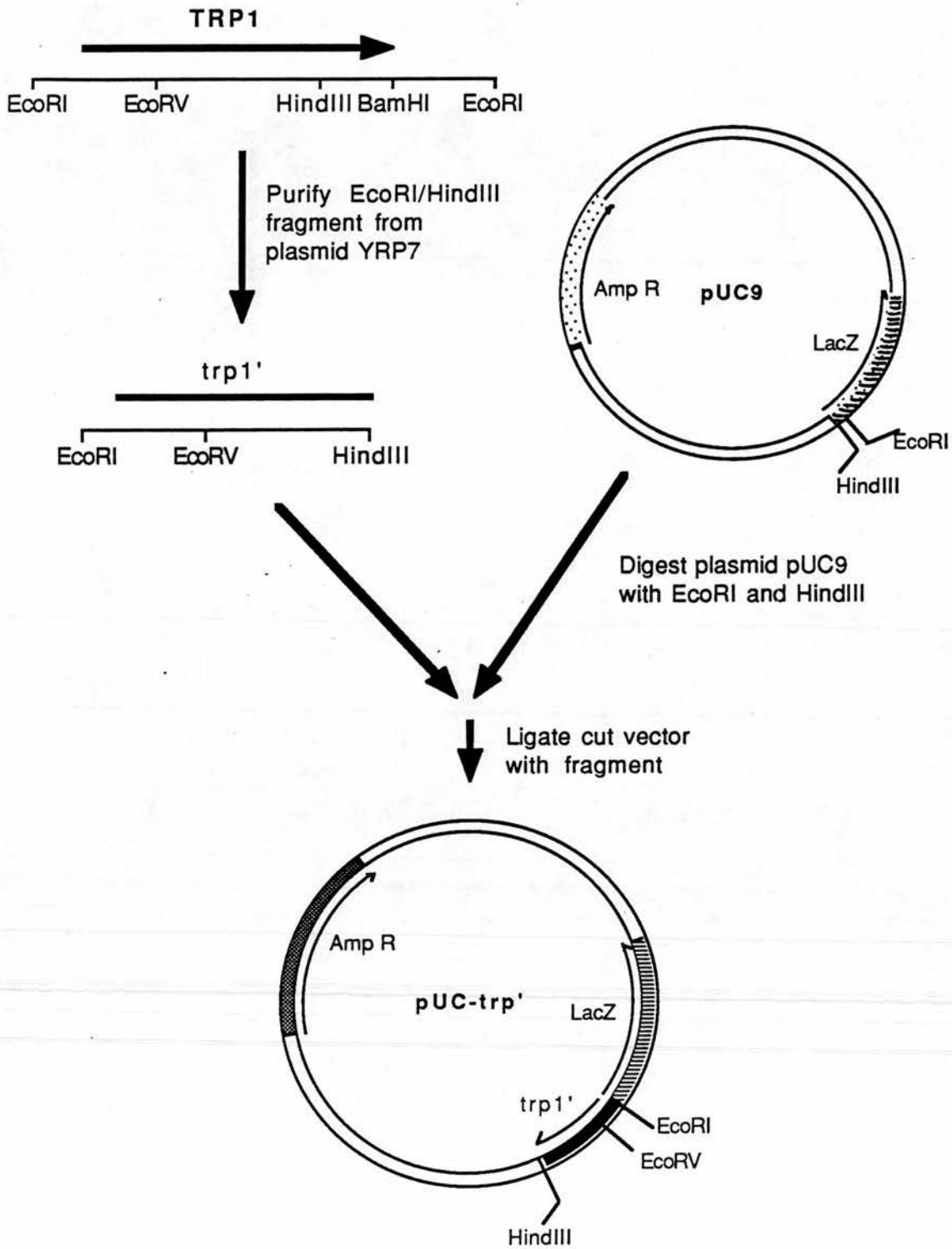
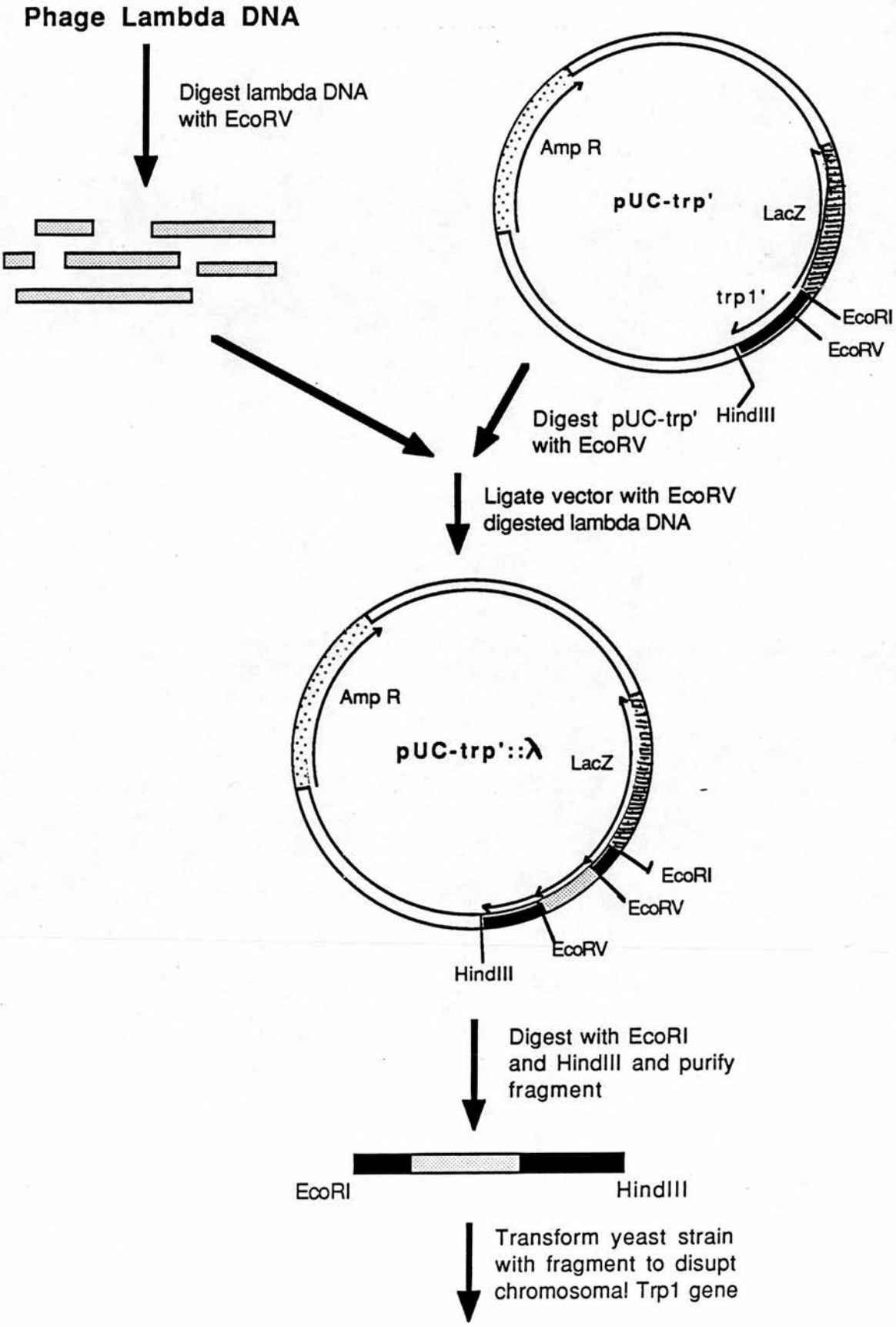

TRP1
EcoRI
EcoRV
HindIII
BamHI
EcoRI
Purify EcoRI/HindIII fragment from plasmid YRP7
trp1'
EcoRI
EcoRV
HindIII
Amp R
pUC9
LacZ
EcoRI
HindIII
Digest plasmid pUC9 with EcoRI and HindIII
Ligate cut vector with fragment
Amp R
pUC-trp'
LacZ
trp1'
EcoRI
EcoRV
HindIII

Fig. 10.2 Disruption of the *TRP1* gene

In order to disrupt the chromosomal copy of *TRP1* to make a Trp$^-$ mutant it was necessary to first disrupt the *TRP1* gene or at least a portion of the gene. It was decided to disrupt the Eco RI/Hind III fragment of the TRP1 gene in pUC-trp' by cloning a piece of disrupting DNA (λ DNA) into the Eco RV site. Phage λ DNA was digested with Eco RV and ligated with Eco RV digested pUC-trp'. The ligation was transformed into NM522 and recombinant plasmids were screened for by restriction analysis (ie an Eco RV digest that released a fragment of λ DNA). A recombinant plasmid was selected that contained a fragment of approximately 1.5 kb of λ DNA (that did not contain Eco RI or Hind III sites) in the Eco RV site of pUC-trp'. The resulting plasmid was named pUC-trp'::λ. The Eco RI/Hind III fragment of pUC-trp'::λ contained a portion of the TRP1 gene disrupted with λ DNA. This fragment was purified and used in to disrupt the chromosomal copy of F102-2 (see text for details).


Phage Lambda DNA
Digest lambda DNA with EcoRV
Amp R
pUC-trp'
LacZ
trp1'
EcoRI
EcoRV
HindIII
Digest pUC-trp' with EcoRV
Ligate vector with EcoRV digested lambda DNA
Amp R
pUC-trp'::λ
LacZ
EcoRI
EcoRV
EcoRV
HindIII
Digest with EcoRI and HindIII and purify fragment
EcoRI
HindIII
Transform yeast strain with fragment to disupt chromosomal Trp1 gene

derived from it was to see whether active toxin is secreted from any of the strains. Active killer toxin could not be detected in the supernatants of any of the strains when standard killer toxin assays were performed (see Materials and Methods). Because of this result it was decided to make sure that the linear plasmids (k1 and k2) encoding the toxin subunits were present in the F102 derivative strains. Unfortunately, no linear plasmids were detected by *in situ* lysis analysis (see Materials and Methods) in any of the strains (data not shown). Therefore it appears as if the strain PWYS4 does not possess the linear plasmids and subsequently the derivatives of this strain will also lack the linear plasmids. In every other respect PWYS4 appears to be the correct strain (ie a derivative of F102-2) as it possesses the correct auxotrophies and lacks Kex2 activity as expected. The most likely explanation for the lack of the linear plasmids in PWYS4 is that the plasmids cannot be stably maintained in the genetic background of PWYS4. It seems possible that production of the toxin polypeptides in a Kex2$^-$ genetic background is deleterious to the cells in some way and thus, cells that lose the linear plasmids are selected for. Cells that have lost the linear plasmids are probably healthier and soon become the majority in a population by outgrowing the linear plasmid containing cells. When strain F102-2 had its KEX2 gene replaced (Chapter 3) the strain contained the linear plasmids. However upon re-examination of the *in situ* lysis gel (fig. 3.3) it was apparent that the strains containing the *kex2::LEU2* disruption do not appear to contain as much linear plasmid as the non-disrupted cells. In hindsight this is possibly due to the linear plasmids not being stably maintained in a Kex2$^-$ genetic background.

10.3 Concluding remarks

Study of the biogenesis of the *K. lactis* killer toxin in cells expressing the different Kex2 constructs was made impossible due to difficulties in obtaining a strain suitable for these studies. The problems were probably due to the instability of the linear plasmids in Kex2$^-$ cells. I suspect that the linear plasmids may also be unstable in other genetic backgrounds such as the *sec1* background (see Chapter 3).

Chapter eleven.

Discussion.

11.0 Discussion.

The amenability of *Saccharomyces cerevisiae* to genetic manipulaton has made it a useful organism for studying many of the basic questions in eukaryote biology. The aim of this project was to use recombinant DNA technology and the powerful genetics of *S. cerevisiae* to facilitate the analysis of the biogenesis of secretory proteins, in particular the *Kluyveromyces lactis* killer toxin since it has a number of interesting features (see Introduction; Chapter 1).

The involvement of the Kex2 endoprotease in the processing of the *K. lactis* killer toxin was confirmed since it was found that strains of yeast which contain the linear plasmids encoding the toxin polypeptides (k1 and k2), but which lack a functional Kex2p, do not secrete any detectable active killer toxin. Furthermore it appears that if Kex2 processing does not occur, the toxin polypeptides are not secreted, even in an unprocessed form (Dr M. Stark personal communication). The Kex2 processing of the αβ toxin precursor was shown to occur at a point in the secretory pathway after that at which the *sec18* mutation (blocking ER to Golgi transport) exerts its effect. This is because at the restrictive temperature of 37°C a *sec18* mutant strain of yeast expressing the toxin (MRY101) accumulated an αβ precursor polypeptide that had not been processed by Kex2p. This observation fits well with the generally accepted view that Kex2p normally processes proteins in the Golgi apparatus.

Another observation was that the toxin subunits do not appear to have been covalently assembled in the ER. This is suprising since the β and γ subunits of the mature toxin are covalently linked by a single disulphide bond and disulphide bonds are widely held to be formed exclusively in the ER; thus it was expected that the αβ precursor and γ subunits accumulated in a *sec18* mutant at the restrictive temperature would co-precipitate in immunoprecipitation experiments. However, this does not appear to be the case as immunoprecipitation experiments showed no detectable co-precipitation of the toxin subunits accumulated in a *sec18* mutant (ie the γ subunit does not appear to be associated with the αβ precursor). The simplest explanation for this observation is that the disulphide bond between the β and γ subunits of the toxin occurs after the point in the secretory pathway at which the *sec18* mutation exerts its effect (from the experiments performed it was not

possible to deduce whether the intramolecular disulphide bonds in the α subunit had been formed or not). The phenomenon of late covalent assembly of polypeptides in the Golgi apparatus has been reported for the assembly of asymmetric acetylcholinesterase (Rotundo 1988) and therefore it is possible that the assembly of killer toxin in yeast may also occur at a late stage in the secretory pathway. The possibility that co-precipitation of the toxin subunits is not detected because of the experimental procedures cannot be ruled out, but as non-reducing conditions were used for extraction and immunoprecipitation of yeast proteins I feel that the likelihood of disulphide bonds between subunits being broken was minimised as far as possible. Another possibility that we cannot rule out is that the lack of co-precipitation of toxin subunits could be a result of the *sec18* mutant being deficient in disulphide bond formation at the restrictive temperature due to changes in the ER. In summary my results suggest that the toxin accumulated in the ER in a *sec18* mutant has not been proteolytically processed by Kex2p, and that disulphide bonds between subunits have not been formed.

Processing events in the secretory pathway occur sequentially in order that secreted proteins are converted into their active secreted form. One of the aims of this project was to alter the timing of the Kex2 processing event in the secretory pathway by localising an active Kex2p to the ER and to investigate the effect of premature Kex2 processing on the biogenesis of secretory proteins. In my attempt to localise an active Kex2p to the ER some interesting observations about retention of proteins in the ER were made. The yeast HDEL retention sequence alone did not appear to be sufficient to facilitate the efficient retention of the Kex2p within the cell since Kex2'-HDEL protein is secreted when expressed at relatively high levels (ie from an episomal plasmid), although when expressed at lower levels (ie from a centromere plasmid) it appears to be efficiently retained. This could be due to the Kex2'-HDEL protein saturating the HDEL retention system when expressed at relatively high levels. However a Kex2'-PDI hybrid protein appears to be much more efficiently retained even when expressed at similar levels, although some is secreted in an apparently clipped form. It is possible that the HDEL element in Kex2'-HDEL is masked and not efficiently recognised by the putative HDEL receptor, but that the addition of the 60 C-terminal amino acids of yeast PDI places HDEL in a natural context and allows the HDEL sequence to be more efficiently recognised. It is also possible that something in the C-terminus of PDI other than the HDEL sequence is

responsible at least in part for the efficient retention of the Kex2'-PDI hybrid protein. Examination of the 60 C-terminal amino acid residues of yeast PDI and yeast BiP (both luminal ER proteins) reveals that they have a very high net negative charge (-19 and -18 respectively; see fig. 11.1). It has been suggested that the ER is made up of a gel matrix of abundant ER proteins (reticuloplasmins; Booth and Koch 1989) and that calcium ions are necessary to stabilise this matrix. It therefore seems reasonable to suppose that the high negative charges at the C-termini of yeast reticuloplasmins PDI and BiP (and possibly other reticuloplasmins) may interact with the positive charge on calcium ions to stabilise a gel matrix. The more efficient retention of Kex2'-PDI might thus be due to the fact that the Kex2'-PDI protein can be stabilised in a gel matrix whereas the other Kex2'p constructs including the Kex2'-HDEL protein cannot. Thus Kex2'-PDI may avoid the normal fate of soluble secretory proteins by being incorporated into this gel matrix. If, when vesicles bud, they only entrap proteins present in the fluid phase, the Kex2'-PDI hybrid protein will not usually be present in the budding vesicles, whereas the other, fully soluble Kex2 constructs will be. The HDEL retention system has been reported to be easily saturable (Pelham *et al* 1988, Dean and Pelham 1990). Therefore the Kex2'-HDEL protein, when expressed at high levels (ie from the episomal plasmids), will probably leave the ER in fairly large amounts, perhaps enough to saturate the putative HDEL receptor that retrieves HDEL-containing proteins from a post ER compartment. In contrast, if the above argument is true, the amount of Kex2'-PDI protein leaving the ER even when the protein is expressed at high levels, will probably be much lower, since most would be present in an immobilised form. This low amount of Kex2'-PDI hybrid protein would probably not be enough to saturate the putative HDEL receptor, allowing it to be recycled back to the ER. When expressed at low levels (ie from the centromere plasmid) the amount of Kex2-HDEL protein, although it leaves the ER is probably not enough to saturate the putative HDEL receptor and this is why no detectable Kex2 activity is present in strains expressing Kex2'-HDEL from centromere plasmids.

Results obtained from the attempts to localise an active Kex2' polypeptide to the ER are thus consistent with the involvement of a mechanism other than the HDEL retrieval mechanism for localising reticuloplasmins to the ER. The model I propose for the retention of reticuloplasmins based on my experimental data is consistent with the model proposed by Booth and Koch (1989) and is as follows:- The negative

Fig. 11.1 The C-termini of yeast PDI and yeast BiP

The C-terminal amino acid (59 residues) sequences of the yeast PDI and yeast BiP proteins are shown. The asterisks indicate the amino acids, aspartic acid (D) and glutamic acid (E) that are negatively charged at pH 7.0. The C-terminus of PDI has 24 negatively charged amino acid residues and carries a net negative charge of -19. The C-terminus of BiP has 22 negatively charged amino acid residues and carries a net negative charge of -18. The reason 59 C-terminal amino acid residues of each of the proteins were looked at was that 59 amino acid residues of yeast PDI are present in the Kex2-PDI hybrid protein.

C-terminus of yeast PDI

```
    *       *       *           *   *
S L D S L F D F I K E N G H F D V D G K

      * *     *       * *   *   *   *
A L Y E E A Q E K A A E E A E A D A E A

* * *   *   *     * * * *       * *
E E D A D A E L A D E E D A I H D E L
```

C-terminus of yeast BiP

```
* *     *         * *   * *     *
D D N F E T A I A E D F D E K F E S L S

                                  *
K V A Y P I T S K L Y G G A D G S G A A

*   * * * * * * * *   *     *   * *
D Y D D E D E D D D G D Y F E H D E L
```

KEY

* negatively charged amino acids at pH 7.0

charge on the C-terminus of PDI (and other reticuloplasmins) is involved in a bulk sorting mechanism i.e. the formation of a gel matrix possibly stabilised by calcium ions. HDEL-containing proteins that escape the bulk sorting and leave the ER are retrieved by the HDEL receptor recycling mechanism which is a backup to the bulk sorting that stops the majority of reticuloplasmins from being secreted. The HDEL-based recycling mechanism probably does not have to deal with large amounts of HDEL-containing proteins and therefore it does not normally matter that it is not capable of dealing with large amounts of HDEL-containing proteins. The bulk sorting model can explain the apparent anomaly that the very abundant proteins BiP and PDI do not normally get secreted even though the HDEL retention system seems easily saturable in yeast.

Another interesting observation arising from the attempts to localise the Kex2' polypeptides to the ER was that although the Kex2'-PDI hybrid protein is the most efficiently-retained construct by far, there was still Kex2 activity secreted from strains expressing Kex2'-PDI. When the secreted Kex2'-PDI polypeptide was compared to the internal (i.e. retained) Kex2'-PDI polypeptide it was discovered to be approximately 4K smaller. This suggests to me that the Kex2'-PDI secreted has been specifically cleaved by a proteolytic enzyme as the secreted polypeptide runs as a distinct band and not as a smear which could possibly arise due to proteolytic degradation. The most likely explanation for the secreted polypeptide being smaller than the internal form is that the C-terminus has been removed by a cleavage somewhere in the PDI portion of the Kex2'-PDI hybrid protein. This cleavage would remove the majority of the PDI portion involved in the putative bulk sorting mechanism of retention and also the HDEL sequence. The cleaved protein would therefore have little or no means of being retained within the cell either by the bulk sorting proposed above or by the specific HDEL recognition mechanisms, and would get secreted along with other secretory proteins. One of the questions that arises from this observation is; what is the enzyme responsible for the cleavage of the Kex2'-PDI protein? From the experiments performed there is no evidence for a vacuolar protease being responsible, i.e. in a strain deficient in vacuolar proteases Kex2'-PDI still gets secreted. Clearly further experiments will be required to determine the reason for this proteolytic cleavage. One interesting possibility is that an ER protease normally involved in removal of aberrant proteins is responsible, analogous to that reported in mammalian cells (see Hurtley and Helenius 1989).

Finally, it is unfortunate that technical problems prevented the analysis of the biogenesis of the *K.lactis* killer toxin processed by the different Kex2' constructs. However, the analysis of the processing of pre-pro α factor by the different Kex2'p constructs has yielded some interesting results. The most striking of these results is that the Kex2'-PDI hybrid protein, which is efficiently retained within the cell and is most likely to be permanently located in the ER, did not process α factor with anything near the same efficiency as the other constructs, although it is known that the Kex2'-PDI protein is fully active in an *in vitro* assay. It is noteworthy that the intracellular level of Kex2'-PDI activity (*in vitro*) is higher than for any of the other Kex2' constructs. There are a number of possibilties for the relatively low *in vivo* activity:- (1) Although the Kex2'-PDI protein can process a small peptide substrate in an *in vitro* assay this does not mean that it will be able to process the larger pre-pro α factor polypeptide efficiently. (2) It is possible that the environment in the ER with respect to pH, ion concentrations etc is not favourable for Kex2 protease activity and therefore very little α factor is processed. (3) If pre-pro α factor is processed in the ER by Kex2p active α factor may not reach the cell surface due to degradation in the secretory pathway. A final possibility exists which arises from the suggestion made above that Kex2'-PDI may be incorporated into the reticuloplasm. If this is the case, pre-pro α factor which is likely to be in the fluid phase, may only rarely come into contact with the Kex2 enzyme and therefore may not be as efficiently processed. The other Kex2' constructs are probably in the fluid phase and can come into contact with the pre-pro α factor substrate and process it more efficiently. Even the Kex2'-HDEL construct, that may be being recaptured by the HDEL retrieval mechanism is probably present in the fluid phase and is in contact with the prepro α factor substrate.

In summary a number of interesting observations have been made regarding the biogenesis of proteins in the secretory pathway and retention of reticuloplasmins in yeast. Additional work will be required to re-inforce some of the observations and hopefully clarify some of the questions that have arisen from this project.

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